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STUDIES ON AVIAN ENCEPHALOMYELITIS. II IMMUNE RESPONSES TO VACCINATION PROCEDURES* †

By B. W. CALNEK and H. JEHNICH

Department of Veterinary Science
University of Massachusetts
Amherst, Massachusetts

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FOR many years following the first reports on avian encephalomyelitis (AE), it was thought that control measures were not feasible.[5] More recent reports, however, suggest that control measures may be not only feasible but also practical.[7,8]

Although chicks infected via egg transmisson are afflicted with ataxia, tremor, and paralysis, breeder hens producing such chicks usually show few or no signs of disease. Yet these hens have been shown to be immune following the infection.[9] Growing or adult birds infected experimentally, on the other hand, do develop central nervous system disturbances as seen in naturally infected chicks.[2]

Not all birds experimentally inoculated are clinically affected with AE. Olitsky injected chicks with AE virus by several routes.[4] Of these, only the intracerebral route con-

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sistently induced signs in 100 percent of the birds. Intradermal, intramuscular, intraperitoneal, intrasciatic, intravenous, and subcutaneous routes of inoculation each produced signs of AE in 20 to 50 percent of inoculated chicks. When birds were inoculated per os, intranasally, or intraocularly, no signs of AE were observed. It was not reported whether survivors were tested for immunity in any manner.

Schaaf and Lamoreux vaccinated large numbers of birds by wing-web inoculation with live virus.[8] The number of birds that developed signs of AE varied from none to 6 percent in different groups. In this case, chickens thus inoculated and showing no clinical infection were tested for immunity and were found to have some resistance to intracerebral challenge.

In a later report,[7] Schaaf indicated that postvaccination signs were seen in as many as 16 percent of chicks inoculated intramuscularly. Per os dosage of AE virus elicited no symptoms in birds, yet induced partial protection against intracerebral challenge with the AE virus.

The observation that birds can develop some immunity without necessarily having clinical signs of AE prompted the work reported here. Particular emphasis was placed on methods of exposure and dosage of AE virus in an attempt to find a method suitably nonpathogenic yet immunogenic for use in vaccination.

MATERIALS AND METHODS

Virus. The strain of AE virus used in these trials was egg-adapted by yolk-sac inoculation of 6-day embryos as described.[1] It was stored as a 1 in 5 (in nutrient broth) suspension of brain material at –20 C.

Virus titration and serum-neutralization tests. Titrations were done by injecting tenfold serial dilutions of the virus into the yolk sacs of 6-day embryos. Embryos were examined 12 days postinoculation for evidence of infection and 50 percent endpoints (EID_{50}) were calculated according to the method of Reed and Muench.[6] Serum-neutralization (SN) tests were conducted as described[1] using whole serum or plasma and tenfold serial dilutions of the virus. A neutralization in-

dex (NI) for each plasma sample was calculated as the difference between the EID_{50} of virus control eggs and the EID_{50} of the virus-plasma titration. The neutralization indices of the birds within a group were averaged to obtain the geometric mean titer (GMT) and the standard deviation of the mean ($s_{\bar{x}}$) was determined. Together they were expressed as $GMT \pm s_{\bar{x}}$

Experimental chickens and eggs. All birds used in this experiment were progeny either of a flock of Rhode Island Red breeders maintained at the University or of a privately owned flock of White Leghorns. Chicks were hatched and raised in isolation units. All birds were held in wire batteries. Eggs used for virus propagation, titrations, and SN tests came from two privately owned flocks of White Leghorns, both of which supplied eggs susceptible to the AE virus.

Vaccination procedures. Intramuscular inoculations were done by injecting 0.1 ml of virus suspension into the thigh with a ¾ inch 25 gauge needle. For wing-web inoculations, a two-pronged needle of the type commonly used for Newcastle disease or fowl pox vaccinations was employed. Approximately 0.01 ml of virus suspension was delivered to each bird by thrusting the needle (after dipping in virus suspension) through the web of the wing.

Vaccination by the spray method was done with the aid of a nebulizer (DeVilbis). A 1:10 suspension of virus in broth was sprayed over the heads of birds held in batteries. Since the actual intake of virus by the birds was not determined, dosage is reported as the amount of virus delivered in the form of a spray.

Water vaccine was applied as a 1:100 virus suspension (in distilled water). It was given as drinking water for a period of 2 hours following 4½ hours of water deprivation. An excess was supplied and the average consumption per bird determined. Per os administration consisted of pipetting virus suspension (in broth) directly into the crops of birds.

Challenge. Birds were challenged after vaccination by intracerebral inoculation. One tenth ml of virus suspension

was injected into the cerebrum by passing a needle either through the foramen magnum or through the bone of the orbit as described by Schaaf.[7] In all instances excepting trial I, the challenge dose consisted of 10,000 EID_{50}. Most birds were totally incapacitated for a short while (2 to 4 hours) following this technique and some never completely recovered. The latter had torticollis or paresis. All birds not completely normal by 6 days after challenge were discarded from the experimental groups. Previous experience indicated the incubation period of AE following intracerebral inoculation to be greater than 6 days.

Criteria of clinical infection following vaccination or challenge with AE. All birds were examined daily. Those that developed ataxia, paralysis, or other signs were removed for post mortem and histopathological examinations. An affected bird was considered to have clinical manifestations of AE infection if gross examination revealed no visible lesions and microscopic examination revealed lesions typical of those described for AE.[3]

RESULTS

Vaccination Trial 1. This trial involved six groups of fifty 6½-week-old chickens. Each of four groups was inoculated with embryo-propagated AE virus (pooled brain suspensions of 8th to 13th passages) via one of the following routes: intramuscular, wing-web, spray, or drinking water. A fifth group was injected intramuscularly with a suspension of non-infectious brain material, while the sixth group served as uninoculated controls.

All birds were observed daily for a 4-week postinoculation period. The number of birds developing signs of AE infection following inoculation was greatest (40 percent) in the group vaccinated by the wing-web route. Other groups were affected as follows: spray—19 percent, intramuscular—14 percent, and drinking water—none. No affected birds were observed in either of the control groups. Birds affected with AE showed first clinical signs between the 13th and 23rd days postinoculation. Table 1 contains detailed data regarding dosage and the appearance of clinical signs.

TABLE I

Clinical signs of AE infection observed following inoculation of 6½-week-old chicken with AE virus by various routes

Group	Route of inoc.	Amount and dilution inoculum per bird	Virus dosage per bird EID_{50}	Signs AE postvacc.	Appearance 1st signs (days postinoc.)
I	Intramus.	0.1 ml 10^{-0}	50,000	7/50* (14%)	13 - 15
II	Wing-web	0.01 ml 10^{-0}	5,000	20/50 (40%)	13 - 21
III	Spray	1 ml 10^{-1}	50,000**	9/47 (19%)	20 - 23
IV	Drinking water	32 ml 10^{-2}	160,000	0/50	
V	Intramus.	0.1 ml Sterile brain 1:5	None	0/50	
VI	None	———	None	0/50	

* Number of birds clinically affected/number vaccinated.
** Amount of virus delivered as aerosol/bird

Serum-neutralization tests were conducted on blood samples from ten birds at 4 weeks postinoculation. Results of SN tests are found in table 2. The neutralization indices of both the wing-web and the intramuscularly inoculated groups had risen significantly by 4 weeks postinoculation. The groups inoculated via spray and drinking water both had slightly elevated neutralization indices. They were not sufficiently elevated to be clearly significant. However, the neutralization indices of the two control groups did not vary significantly throughout the course of the experiment.

TABLE II

Serological responses following inoculation of 6½-week-old chickens with AE virus by various routes. Vaccine trial 1.

Group	Inoculum	10 birds Prevacc.	5 birds 2 weeks Postvacc.	10 birds 4 weeks Postvacc.
I	AE virus Intramuscular	.02±.10* (−.6 to .4)	2.20±.30 (1.4 to 3.1)	2.60±.13 (1.8 to 3.4)
II	AE virus Wing-web	−.03±.11 (−.5 to .4)	1.40±.19 (.5 to 1.5)	1.98±.05 (1.5 to 2.2)
III	AE virus Spray	−15±.10 (−.4 to .5)	−.04±.08 (−.2 to .2)	.54±.11 (−.1 to 1.1)
IV	AE virus Water	−.14±.16 (−.7 to .8)	.76±.19 (.3 to 1.4)	.65±.11 (.2 to 1.1)
V	Sterile brain Intramuscular	.16±.14 (−.6 to .8)	.42±.13 (−.1 to .7)	−.06±.11 (−.7 to .4)
VI	None	.06±.08 (−.5 to .3)	.24±.14 (−.1 to .7)	.08±.06 (−.4 to .8)

* Geometric mean titers ± standard deviation of the mean. Numbers in parentheses indicate the range of neutralization indices within the group.

At 4 weeks postinoculation, the remaining birds in each group were challenged by intracerebral inoculation of 11th passage embryo propagated AE virus. Using tenfold serial dilutions of the virus, a titration was done in each group. Results of the challenge are found in table 3. All of the birds in the wing-web and intramuscular groups withstood the challenge. In the two control groups, ID_{50} titers of $10^{-4.4}$ and $10^{-4.6}$ were observed. In the spray and drinking water groups

ID_{50} titers of $10^{-3.2}$ and $10^{-3.4}$ respectively were attained. It is seen in table 3, however, that in the latter two groups, many birds survived the low dilutions while others succumbed to high dilutions. This would suggest that immunity levels in these two groups were not consistent.

TABLE III

Mortality observed following intracerebral challenge of birds 4 weeks after inoculation with AE virus by various routes. Vaccine trial 1.

	Group and Vaccination					
Virus Dilution	I AE I.M.	II AE Wing-web	III AE Spray	IV AE Water	V Ster. Brain I.M.	None
10^{-1}	0/7*	0/5	1/3	3/6	7/8	6/6
10^{-2}	0/4	0/4	3/5	3/5	5/5	4/4
10^{-3}	0/7	0/4	4/6	3/5	8/8	3/3
10^{-4}	0/5	0/4	2/3	3/5	5/5	5/5
10^{-5}	0/5	0/5	1/1	3/6	0/6	1/5
ID_{50}	10^{-1}	10^{-1}	$10^{-3.2}$	$10^{-3.4}$	$10^{-4.4}$	$10^{-4.6}$

* Number of birds that succumbed to AE challenge/number challenged.

Vaccination Trial 2. Five groups of ten 7-week-old birds were used in trial 2. Two vaccinations were applied with a 4 week interval. For the first vaccination, Groups I and V received no vaccine, while Groups II, III and IV were dosed with a drinking water vaccine. This was applied as a 10^{-2} dilution of virus in distilled water and was given for 2 hours following 4½ hours water deprivation. The average dosage per bird was 2,500,000 EID_{50} of embryo propagated AE virus (pooled virus from 4th to 20th passages). For the second vaccination, Groups I and II received no vaccine, Group III was dosed a second time with water vaccine (300,000 EID_{50} AE virus; 21st embryo passage), while Groups IV and V were vaccinated via the wing-web with 6,000 EID_{50} per bird.

Daily observations were made on all groups. No clinical signs of AE infection were observed in this trial.

Serum-neutralization tests were done on samples from five birds in each group just prior to vaccination and at 4, 8, 11 and 18 weeks after vaccination. The results of these tests are presented in table 4. Vaccine applied via the drinking water

induced substantial increases in neutralization indices in some birds, whereas no rise was seen in others. Of the 15 birds (groups II, III and IV) tested 4 weeks after the 1st dose of water vaccine, nine had neutralization indices between 1.2 and 3.0; the other six ranged from 0.3 to 1.1. Wing-web vaccine, whether applied alone, or following a water vaccination, stimulated neutralization indices ranging between 1.1 and 3.8. Titers in birds dosed two times with water vaccine ranged between 1.2 and 3.9 at 4 weeks after the second vaccination. Group I birds (negative controls) had no neutralization indices above 0.6 throughout the trial.

At 18 weeks after the 1st vaccination, all remaining birds were challenged intracerebrally. Each bird received approximately 10,000 EID_{50} of the 20th embryo passage AE virus. All birds survived this challenge except the negative controls (Group I) of which two out of three succumbed to AE infection.

Vaccination Trial 3. This trial consisted of intramuscular vaccination of five groups of ten 8-week-old chickens with graded doses of AE virus (21st embryo passage). Tenfold dilutions of the virus in broth were made to give individual doses of 150,000 to 15 EID_{50} per 0.1 ml. A sixth group of five birds served as uninoculated controls and were held in the same isolation unit but in a separate battery. Serum-neutralization tests were conducted on five birds per group just prior to vaccination and again at 4 weeks postvaccination. At 4 weeks postvaccination, each of the remaining birds was challenged intracerebrally with 10,000 EID_{50} of 20th embryo passage AE virus. All birds with clinical signs were examined for evidence of AE infection in the manner described above. Results of this trial are given in table 5. It is seen that within each group, roughly equal numbers of birds (about 30 percent) succumbed to the vaccination. Although the postvaccination reaction did not vary significantly with the dosage, the serological response was greatest with the largest inoculum and least with the smallest. In the group receiving 150,000 EID_{50} all of the neutralization indices exceeded 2.1

TABLE IV

Neutralization indices following vaccination of 7-week-old birds with AE virus via the drinking water or by wing-web inoculation. Vaccine trial 2.

Group	Vaccination		Serological response				
	1st Vacc.	2nd Vacc. (4 wks.)	Prevacc.	4 wk	8 wk	11 wk	18 wk
I	None	None	.42±.06* (.3 to .5)	.16±.15 (−.4 to .5)	.14±.17 (−.5 to .5)	.12±.12 (−.2 to .5)	−.26±.17 (−.7 to .3)
II	Water vacc.** 2,500,000 EID_{50}	None	.34±.12 (.1 to .7)	1.60±.59 (.3 to 3.0)	1.75 (1.7 to 1.8)	2.00 (1.5 to 2.5)	1.35 (1.0 to 1.7)
III	Water vacc. 2,500,000 EID_{50}	Water vacc. 300,000 EID_{50}	.20±.21 (−.4 to .6)	1.60±.45 (.7 to 2.8)	2.20±.80 (0 to 3.5)	2.22±.53 (1.2 to 3.9)	1.68±.36 (1.0 to 2.8)
IV	Water vacc. 2,500,000 EID_{50}	Wing-web vacc. 6,000 EID_{50}	−.12±.09 (−.5 to 0)	1.70±.08 (.9 to 2.4)	2.64±.45 (1.4 to 3.7)	2.22±.51 (1.1 to 3.8)	2.02±.45 (0.3 to 2.8)
V	None	Wing-web Vacc. 6,000 EID_{50}	.68±.06 (.2 to 1.0)	.38±.39 (0 to 1.0)	3.08±.29 (2.0 to 3.5)	2.74±.31 (2.1 to 3.3)	2.02±.45 (0.8 to 3.7)

* Geometric mean titer ± standard deviation of the mean. Based on 5 birds per group except for Group II at the 8, 11, and 18 week bleedings where only two birds were tested. Numbers in parentheses indicate the range of the neutralization indices within the group.

** Route of administration and virus dosage/bird.

with an average NI of 2.9. Those from the group vaccinated with 15,000 EID_{50} were slightly more variable (average NI 2.2, range 1.5 to 3.1). The birds in the third group (1,500 EID_{50}) had consistently lower responses. In this case the range was 1.1 to 1.6 with the average NI being 1.3. Birds inoculated with 150 EID_{50} were inconsistent in their response, as were those receiving 15 EID_{50}. In the former, two birds remained negative (NI of 0.2) whereas three birds were positive (NI range of 1.9 to 3.2). The latter group had only two of five birds developing significant neutralization indices (1.5 and 2.3); the other three birds all had indices less than 0.9. Neutralization indices in the negative controls never exceeded 0.5.

During a four week period following intracerebral challenge, 100 percent of the negative controls developed ataxia and paralysis due to AE infection. All of the vaccinated birds survived the challenge with no ill effects.

TABLE V

Clinical infection, serological response and resistance to intracerebral challenge following intramuscular vaccination of 8-week-old chickens with live AE virus. Vaccine trial 3.

Group	Virus dose per bird EID_{50}	Signs AE Postvacc.	Serological response		Signs AE following intracerebral challenge
			Prevacc.	4 wks Postvacc.	
I	150,000	3/10*	.16±.17** (−.2 to .7)	2.88±.18 (2.1 to 3.2)	0/4
II	15,000	3/10	.10±.12 (−.2 to .5)	2.44±.58 (1.5 to 3.1)	0/4
III	1,500	3/10	.45±.35 (−.2 to .9)	1.25±.10 (1.1 to 1.6)	0/5
IV	150	3/10	.34±.20 (.1 to .7)	1.54±.58 (.2 to 3.2)	0/5
V	15	2/10	.22±.22 (−.3 to .8)	1.08±.34 (0 to 2.3)	0/6
VI	None	0/5	−.18±.14 (−.7 to .1)	.16±.14 (−.1 to .5)	4/4

* Fractions indicate the number of birds with clinical signs of AE/ number of birds vaccinated or challenged.

** Geometric mean titer ± standard deviation of the mean. Based on 5 birds per group. Numbers in parentheses indicate the range of neutralization indices within the group.

Vaccination Trial 4. Five groups of ten 7-week-old birds were used to test the effect of dosage on pathogenicity and immune response following wing-web inoculation. The vaccine virus and experimental plan employed were the same as those used in trial 3. Tenfold serial dilutions resulted in doses of 15,000 to 15 EID_{50} per .01 ml. One group was held as uninoculated controls. Serum titers were done prior to vaccination and at 4 weeks postvaccination. All surviving birds were challenged by intracerebral inoculation with 10,000 EID_{50} at 4 weeks after vaccination. Detailed data and results are found in table 6. Clinical signs of AE were observed in all four inoculated groups. The only group in which a consistent serological response was elicited was that which had recived 15,000 EID_{50}. Neutralization indices in this group ranged from 1.7 to 2.6 with an average of 2.2. In the other three groups, the responses were lower and more variable. Only the top dosage (15,000 EID_{50}) protected all birds against intracerebral challenge. Controls had no immune response and were all susceptible to challenge.

TABLE VI

Clinical infection, serological response and resistance to intracerebral challenge following wing-web vaccination of 7-week-old chicks with live AE virus. Vaccine trial 4.

Group	Virus dose per bird EID_{50}	Signs AE Postvacc.	Serological response		Signs AE following intracerebral challenge
			Prevacc.	4 wks Postvacc.	
I	15,000	3/9*	.16±.24** (−.5 to .7)	2.20±.34 (1.7 to 2.6)	0/4
II	1,500	2/9	.32±.17 (−.1 to .7)	.66±.10 (.4 to 1.1)	1/4
III	150	1/9	.48±.10 (0 to .7)	.76±.39 (0.1 to 2.1)	2/4
IV	15	1/8	.28±.15 (−.1 to .6)	.38±.17 (0 to 1.0)	2/4
V	None	0/10	.34±.10 (0 to .6)	.26±.16 (−.2 to 7)	3/3

* Fractions indicate the number of birds with clinical signs of AE/number of birds vaccinated or challenged.
** Geometric mean titer ± standard deviation of the mean. Based on 5 birds per group. Numbers in parentheses indicate the range of neutralization indices within the group.

Vaccination Trial 5. When graded doses of AE virus were administered per os to three groups of ten 7-week-old birds, no clinicial signs were observed. This trial was conducted in the same manner as trials 3 and 4 with dosages ranging from 1,500,000 to 15,000 EID_{50} per bird. The virus suspensions were pipetted directly into the crops of the birds, each bird receiving 1.0 ml. Only the group dosed with 1,500,000 EID_{50} demonstrated any immunity following vaccination; neutralization indices averaged 1.66 (1.0 to 2.5) and six of seven were protected against challenge with 10,000 EID_{50} at 4 weeks after vaccination. Lower doses elicited no serological response and there was no significant difference in susceptibility to intracerebral challenge between these groups and the controls. Results are found in table 7.

TABLE VII

Clinical infection, serological response, and resistance to intracerebral challenge following per os vaccination of 7-week-old chicks with live AE virus. Vaccine trial 5.

Group	Virus dose per bird EID_{50}	Signs AE Postvacc.	Serological response		Signs AE following intracerebral challenge
			Prevacc.	4 wks Postvacc.	
I	1,500,000	0/8*	.40±.04** (.3 to .5)	1.66±.17 (1.0 to 2.5)	1/7
II	150,000	0/10	.40±.17 (0 to .8)	.22±.14 (−.2 to .4)	6/9
III	15,000	0/9	.26±.26 (.1 to .7)	.54±.14 (0 to .9)	6/8
IV	None	0/10	.24±.17 (−.3 to .7)	.32±.06 (.2 to .4)	7/9

* Fractions indicate the number of birds with clinical signs of AE/ number of birds vaccinated or challenged.

** Geometric mean titer ± standard deviation of the mean. Based on 5 birds per group. Numbers in parentheses indicate the range of neutralization indices within the group.

DISCUSSION

It is evident from these results that both route of administration and dosage of virus are important factors to be considered in the development of an immunization program against avian encephalomyelitis. With either the intramus-

cular or wing-web method of vaccination, a satisfactory immune response could not be produced without the appearance of clinical signs in some birds. It appeared that the percentage of ataxic birds following vaccination by these two methods was not greatly dependent upon the dosage of virus (within the limits used in these trials).

The immune response, as interpreted by the SN test, showed a more definite relationship to virus dosage than did postvaccination reaction. The administration of 15,000 or more EID_{50} produced consistently high neutralization indices (usually above 2.0) when given by the wing-web or intramuscular methods. As dosage was lowered, the neutralization indices became inconsistent as well as lower.

AE virus administered by the spray method (trial 1) caused ataxia in 19 percent of the birds thus vaccinated. Although pathogenicity was relatively high, the serological response was slight. No neutralization indices above 1.1 were detected at 4 weeks after vaccination and very little resistance to intracerebral challenge was demonstrated in this group.

Of the methods employed in this study, only the oral route of administration, either as a drinking water vaccine or per os dosage, permitted the immunization of birds against AE without the appearance of some clinical signs. The amount of virus required to produce a significant serological response, however, was greatly in excess of that needed for wing-web or intramuscular vaccination. It would appear that 10^6 or more EID_{50} per bird are necessary, when given by the oral route, to give a reasonable amount of protection.

It should be noted that in many instances birds having no detectable neutralizing antibodies (NI of less than 1.1) were protected against intracerebral challenge with as many as 10,000 EID_{50} AE virus. On the other hand, no bird having an NI of 1.1 or greater succumbed to challenge. The latter lends support to the use of the SN test as a means of determining immune responses and as a scale by which to compare and evaluate various vaccination procedures.

In all of the experiments included in this report, the birds

were held in batteries with wire floors. The possibility that AE may be essentially an infection of the intestinal tract and that ingestion may be the primary means of spread should not be overlooked. If such be true, in all probability a lower dosage of AE virus by the oral route or the inoculation of a small percentage of the birds within a group would make this means of immunization extremely feasible for floor raised birds.

The virus employed in these trials was an extremely pathogenic one. Perhaps the selection of a strain of virus less virulent would make the intramuscular or wing-web route of inoculation more suitable for immunization. It would appear that the strain used by Schaaf[7] was less pathogenic than that reported here and, by the same token, even less virulent strains doubtless exist.

SUMMARY

1. The virus of avian encephalomyelitis was administered in various dosages to 6½ to 8-week-old chickens as spray, oral, wing-web and intramuscular vaccines.

2. Pathogenicity following vaccination was observed to be rather sharp (as high as 40 percent in some instances) following all methods except oral administration (no clinical signs). The dosage did not greatly influence the degree of pathogenicity.

3. The immune response as determined by the SN test and by resistance to intracerebal challenge depended upon the dosage. Consistently good serological responses were observed following wing-web or intramuscular inoculation of 10^3 or more embryo infective doses (EID_{50}). Oral administration required 10^6 or more EID_{50} for an adequate response.

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THE DEVELOPMENT OF A CHICKEN EMBRYO LETHAL STRAIN OF AVIAN ENCEPHALOMYELITIS VIRUS

By R. W. MOORE and A. I. FLOWERS

Department of Veterinary Microbiology
Texas Agricultural Experiment Station
College Station, Texas

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AVIAN encephalomyelitis was first reported in the United States by Jones in 1930[1]. Since the first report, the disease has occurred sporadically wherever chickens are raised. The clinical signs of the disease occur in chicks. It produces an ataxia or a tremor of the head and neck or both (epidemic tremor). Affected chickens that are isolated from other birds infrequently die. Deaths that do occur are probably due to inanition or trampling. The economic loss from this disease

is not great but it does cause concern to the hatcheryman and the producer. The incidence of this disease has apparently increased in recent years.

In 1955, Schaff and Lamoreux[3] suggested a vaccine for potential breeders to prevent the development of clinical signs in young chicks. Taylor *et al.*[5] described the effect of the disease in breeder flocks and reported that it caused a reduction of hatchability. This effect upon hatchability suggested the approach that was used in this study. It was reasoned that if this agent killed some naturally infected embryos, it might be possible to adapt a strain to kill all embryos by inoculating them during the early stages of embryonic development. The strain used in this study was highly virulent as judged by its effect on hatchability and by the percentage of chicks showing signs of the disease. Since the condition evidently produces embryonic mortality, one-day-old embryos were used for the first few passages.

Jungherr *et al.*[2,4] reported the propagation of a strain of avian encephalomyelitis virus by inoculation into the eye of chicken embryos. The average titer of this virus was 10^{-5} based upon embryonic inactivity and lesions. The virus propagated by this technique was reported to produce dwarfing and embryonic inactivity but little or no embryo mortality.

Wills and Moultrap[6] reported the propagation of avian encephalomyelitis virus in the chicken embryo by yolk sac inoculation. They successfully propagated the strain through 17 serial passages without a reduction of pathogenicity to day-old chicks. Embryo mortality was not a characteristic of this strain of virus.

METHODS

The virus for this study was obtained from 10-day-old chicks with symptoms and microscopic lesions of avian encephalomyelitis. This particular strain of virus caused signs in chicks at the time of hatching and there was a sharp decrease in hatchability such as Taylor *et al.*[5] described.

A 20 percent suspension of chick brain in nutrient broth was inoculated into the yolk sac (0.2 ml) of one-day-old

chicken embryos which were free of avian encephalomyelitis antibodies. The chicken embryos were candled on the fourth day post-inoculation and only viable ones at that time were considered for further study. As embryonic mortality occurred, brain material and yolk fluid was harvested from the dead embryos. Table 1 shows the amounts of inoculum and the age of embryos used in each passage. After the tenth passage, embryo mortality occurred early enough so that the whole embryo could be harvested. By the use of a 15 gauge needle and syringe the embryo was collected and suspended in approximately 5 ml of yolk fluid.

Virus titers were determined at the 10th, 17th, and 20th passage by inoculating 0.2 ml of each dilution into the yolk sac of 5-day-old chicken embryos. At the 21st and 22nd passage, virus titers were determined in parentally immune chicken eggs. On the 20th passage, a serum neutralization test was attempted by inoculating 0.2 ml of each virus dilution and 0.4 ml of serum-virus combination of each dilution into five 5-day-old chicken embryos to determine if the agent was neutralized by a known anti-avian encephalomyelitis serum.

The anti-avian encephalomyelitis serum was prepared by inoculating 16-week-old chickens intramuscularly with 1 ml of a 20 percent brain suspension from chicks infected with the Schaaf strain.[3] Blood was collected one month post-inoculation and the serum separated. The serum was checked for the presence of neutralizing antibodies to avian encephalomyelitis virus, infectious bronchitis virus and quail bronchitis (CELO) virus.

RESULTS

The agent on initial passage killed chicken embryos when they were inoculated into the yolk at one day of incubation. By the fifth passage it killed all of the inoculated embryos (table 1). The age at inoculation of the chicken embryos was increased to six days at the seventh passage and the virus has been repeatedly passed by inoculating into the yolk sac of either five or six-day-old chicken embryos for a total of 20 passages.

TABLE I

The death patterns and inoculums used in the adaptation of a strain of avian encephalomyelitis

No. Passage	Age of embryos in days I/C		Mortality Days following inoculation 1	2	3	4	5	6	7	8	9	10	11	12	13	Remarks
1	10	1					1						1			Chick brain material
2	10	1	1			1										.4 ml inoculum Remainder dwarfed
3	10	4							1	1						.4 ml inoculum
4	10	1												2	1	Added dihydrostreptomcin 25mg/cc Remainder dwarfed .2ml inoculum
5	10	1				2	1			1		6				.2ml inoculum
6	10	4				1	1	1	4			1	1			Remainder dwarfed .4ml inoculum
7	10	6					2	2	2	2	1			1		Added streptomycin 25 mg/ml .4ml inoculum
8	10	6	2			1	1	2		2			1		1	Added streptomycin 25 mg/ml .4ml inoculum
9	5	6	1						2			1	1			.4ml inoculum
10	5	5					4		1							.2ml inoculum
11	10	5		1	1	3	3	2								.4ml inoculum
12	5	6	1			2		1			1					.4ml inoculum
13	5	6					3	2								.4ml inoculum
14	5	6			1	2	1									.4ml inoculum
15	5	6	1		1	2	1									.4ml inoculum
16	5	6	1			3	1									.4ml inoculum
17	5	5			5											.2ml inoculum
18	5	6		3		2										.2ml inoculum
19	5	6			2	3										.2ml inoculum
20	5	5	2	1	1	1										.2ml inoculum

When the agent was titrated at the 10th passage, it did not kill the chicken embryos except with the undiluted virus but dwarfing was observed through a 10^{-5} dilution. By the 17th passage, the agent had become adapted so that it killed embryos through a 10^{-5} dilution. It produced only questionable signs when .02 ml of egg material was inoculated intracerebrally into one-day-old chicks. The 20th passage virus had an ELD_{50} 10^{-5}. When virus titrations were attempted in parentally immune embryonating eggs, titers were depressed by approximately four logs.

The serum neutralizing index of a known anti-avian encephalomyelitis serum was 4 (table 2). This serum did not neutralize either infectious bronchitis virus or quail bronchitis virus indicating that the agent was neutralized only by avian encephalomyelitis antibodies.

TABLE II

A serum neutralization of the adapted 20th passage virus using a known anti-avian encephalomyelitis serum

Dilution	Virus only Death in embryos	Virus and serum Death in embryos
Undiluted	5 of 5	1 of 4
10^{-1}	5 of 5	3 of 4
10^{-2}	5 of 5	0 of 4
10^{-3}	5 of 5	0 of 4
10^{-4}	5 of 5	0 of 4
10^{-5}	3 of 5	0 of 4
10^{-6}	0 of 5	0 of 4

DISCUSSION

This agent should prove to be a good laboratory tool for the detection of antibodies to avian encephalomyelitis. It is quite advantageous in time and effort to have a technic which evaluates by embryo mortality instead of by dwarfing or lesions. The agent described by Jungherr[2,4] had an average titer of 10^{-5} by embryonic inactivity and the average titer of the agent described in this report was 10^{-5} ELD_{50}. The immunizing properties of this agent are unknown but are being evaluated. The ease of laboratory manipulation and lack of pathogenicity in day-old chicks would suggest that this may be an ideal immunizing agent.

SUMMARY

Embryonic mortality was produced by a strain of avian encephalomyelitis after an adaption technic involving the use of day-old embryos for the initial passages. This agent had an ELD_{50} titer of 10^{-5} which was sufficient to allow valid interpretations of a serum neutralization test. The described agent does not produce mortality and only questionable signs when inoculated intracerebrally into day-old-chicks.

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AVIAN ENCEPHALOMYELITIS IMMUNIZATION WITH INACTIVATED VIRUS

By KERMIT SCHAAF
Kimber Farms, Inc., Niles, California

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EFFECTIVE control of avian encephalomyelitis (AE), sometimes referred to as epidemic tremor, has been described in an earlier report[6] from this laboratory. Prospective breeding chickens are vaccinated during their growing period with the live virus to insure development of immunity before eggs are saved for hatching, thus preventing a later outbreak during which the disease might be transmitted through the eggs to their progeny.

This study was undertaken to measure the effectiveness of inactivated virus vaccine designed for use in situations where there is risk that live virus might spread to susceptible breeding hens.

MATERIALS AND METHODS

Host chicks. White Leghorn pullet chicks whose dams had been vaccinated with live virus of AE served as hosts in these experiments. These chicks were reared in buildings separate from other chicks, and were not vaccinated for any other disease. Housing was in floor pens with litter except in experiment 3, where the chicks were kept in single-deck battery brooders throughout the test. Contact control chicks were used in experiment 1. In experiment 2, controls were in the same house with the vaccinated birds, but in a separate pen. In experiment 3, the contact controls were in batteries

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with the vaccinated birds and the isolated (as opposed to contact) controls were in a separate battery in the same room.

Virus. A strain of AE virus described previously was used for these trials. The virus was propagated in chicks which had been inoculated intracerebrally (IC) with virus of the 35th passage. These were destroyed on the second day following the onset of symptoms, and their brains aseptically removed. Collections of brain tissue, amounting to 100 grams or more, were homogenized with equal volumes of sterile distilled water in a blender (Osterizer) for 5 minutes and stored at —30C. The dose lethal to 50 per cent of the test chicks (CLD_{50}) was determined by inoculating groups of chicks with 10-fold dilutions of the virus and calculating the 50 percent end point by the method of Reed and Muench[5]. When these collections of virus in brain tissue were titrated in this manner in susceptible chickens of the same age as the vaccinated chickens at the time they were challenged, with an intracerebral dose of 0.2 ml, they were found to contain from $10^{6.13}$ to $10^{7.11}$ CLD_{50} per gram of brain tissue.

Preparation of vaccine. Beta-propiolactone (BPL) was found by LoGrippo and Hartman[3] to be capable of inactivating several neurotropic viruses in 10 to 15 minutes at 37C, whereas formalin and phenol required days for the same degree of inactivation under parallel conditions. BPL had the additional advantage that the virus vaccines inactivated with it retained a significantly higher degree of antigenicity. Further evidence of the usefulness of BPL has been provided by Mack and Chotisen[4] in their description of an effective inactivated Newcastle disease virus vaccine.

In view of these reports BPL was chosen for inactivating the AE virus used in this study.

BPL is a highly reactive chemical which hyrolyzes in a few hours when in dilute solution, and has acidic end-products. To counteract this, the BPL-inactivated vaccines described by LoGrippo and Hartman[3] were buffered to pH 7.2 - 7.4 before use. Although Mack and Chotisen[4] do not mention any alteration of the acid pH in their work with BPL, it was thought desirable in these tests to buffer some of the trial vaccines.

Since neither of the papers referred to reported trials with vaccines containing adjuvants it seemed worthwhile to explore the possible advantages of an adjuvant in this study.

Vaccine 1 was inactivated by adding 500 ml of chilled 50 percent virus-infected brain tissue in nutrient broth to an equal volume of a 1.0 percent solution of BPL prepared immediately before use in chilled distilled water. This combination was blended in an Osterizer for two minutes and left at room temperature (approximately 20C) overnight to allow complete hydrolysis of the BPL, after which it was stored at 4C. The pH of the brain tissue suspension, which before inactivation was 6.3, dropped to 4.4 after mixing with BPL. To 750 ml of the inactivated virus suspension, 250 ml of a 25 percent aluminum hydroxide suspension was added and the mixture blended in the Osterizer for 3 minutes. After allowing it to remain overnight at 4C., 200ml of 1 molar disodium acid phosphate were added, and the total volume of 1200 ml was blended in the Osterizer for 30 seconds. The final pH was 7.3.

Vaccine 2 was prepared from a different collection of virus-infected brain tissue, inactivated in the same manner as vaccine 1.

Vaccines 3, 4, and 5 were prepared from aliquots of vaccine 2, and represent either different concentrations of inactivated virus-infected brain tissue and aluminum hydroxide or differences in pH. These variables are shown in table 1. All vaccines were stored at 4C until used.

TABLE I

Composition of BPL-inactivated vaccines

Vaccine number	pH	Aluminum hydroxide %	Brain tissue (virus) %	CLD_{50} per ml. (log)
1	7.3	5.2	15.6	5.3
2	4.4	none	25.0	6.5
3	7.3	none	16.6	6.3
4	4.6	6.25	18.7	6.4
5	7.4	6.25	12.5	6.2

Test for virus activity in vaccine. A portion of the BPL-inactivated virus pool used to prepare vaccine 1 was set aside before the addition of aluminum hydroxide. This was diluted with an equal portion of nutrient broth containing 5 mg of streptomycin per ml, and was injected intracerebrally in 0.2 ml doses into twelve 63-day-old chickens. The intracerebral inoculum consisted of 12.5 percent brain tissue, and each intracerebral dose was calculated to contain approximately 1,250,000 CLD_{50} (log $10^{6.1}$) of BPL-inactivated virus material. The inoculated chickens remained normal throughout an observation period of 60 days, indicating that inactivation had been complete.

An attempt was made to conduct a similar test with BPL-inactivated vaccine containing aluminum hydroxide but inclusion of this material in the vaccine caused the immediate death of all chickens into which it was injected by the intracerebral route.

Method of vaccination. All vaccines were injected into the breast muscle. A 5/8 inch 20 gauge needle, fitted to a 10 ml syringe equipped with an adjustable dosage lock nut was used. In experiment 1, graduated doses were given, from 0.1 ml to 1.0 ml to four lots of chickens. In experiments 2 and 3 each vaccinated chicken received 1.0 ml of the vaccine. The chicks in experiments 1, 2, and 3 were 63, 42, and 34 days of age, respectively, when vaccinated.

Method of challenge. The challenge dose for all chickens was 0.2 ml, given intracerebrally with a ¾ inch 20g needle fitted to a 2 ml glass syringe. All test fluids inoculated intracerebrally contained 2.5 mg of streptomycin per ml. In order to avoid clogging the needle with bone, the brain was entered through the thin bone of the orbit, behind the eyeball.

The challenge dose of approximately 257 CLD_{50} was chosen as a convenient dilution of recently titrated virus pools, and was within the range of dosage known from previous tests with this strain of virus to be capable of producing symptoms of the disease in all or nearly all unvaccinated control chickens.

The chickens in experiments 1, 2, and 3 were challenged

37, 36, and 47 days, respectively, after vaccination. Thirteen chickens died immediately from trauma associated with the intracerebral inoculation. These amounted to slightly less than 4 percent of all the chickens in the test, and were excluded from the final tabulations.

RESULTS

The intramuscular injection of BPL-inactivated AE virus vaccine, with or without the addition of aluminum hydroxide, had no apparent ill effect on the chickens. Symptoms of AE appeared as early as 10 days among the challenged control chickens and at 13 days among the challenged groups of vaccinated birds. These incubation periods are within the extremes of 9 to 14 days usually shown by this strain of AE virus when inoculated intracerebrally into susceptible chickens. Most of the chickens which succumbed to challenge did so by the twentieth day. Survivors were continued on experiment, however, until the fiftieth day in experiment 1 and the thirty-fifth day in experiments 2 and 3 to make sure the chickens had ample time to demonstrate their ability to withstand challenge.

In experiment 1, where vaccine 1 was given at 4 dosage levels, only 7 of the 37, or 18.9 percent of the chickens receiving 1.0 ml of the vaccine succumbed to challenge (figure 1). Recipients of smaller doses of vaccine showed less protection but in only one instance did mortality exceed 50 percent. In contrast, 28 of 29 (96.6 percent) of the control chicks died from the challenge dose.

The group of chickens which received the 0.1 ml dose of vaccine survived challenge equally as well as the group which received a vaccine dose 5 times as large. While this appears to be an anomalous result it is not an uncommon finding in tests of this sort.

In experiment 2, (figure 2) acid and alkaline vaccines, each with and without aluminum hydroxide, were compared. Vaccines 4 and 5, containing aluminum hydroxide, were more than twice as effective (regardless of their pH) in preventing mortality from intracerebral challenge than vaccines 2 and 3, which did not contain this adjuvant. Only 4 of 14, or

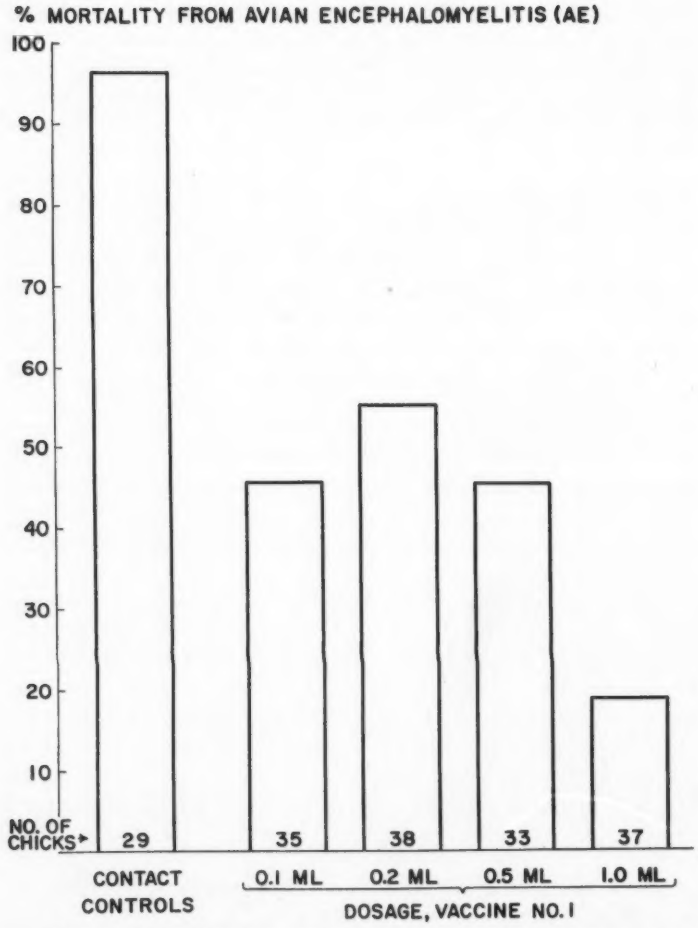

Fig. 1. Mortality from avian encephalomyelitis following intracerebral challenge among controls and among groups of chickens vaccinated intramuscularly with different dosages of vaccine 1 described in table 1.

28.6 percent of the chickens in each of the groups receiving vaccines 4 and 5 succumbed to challenge. Of vaccines 2 and 3, which did not contain aluminum hydroxide, vaccine 3 which

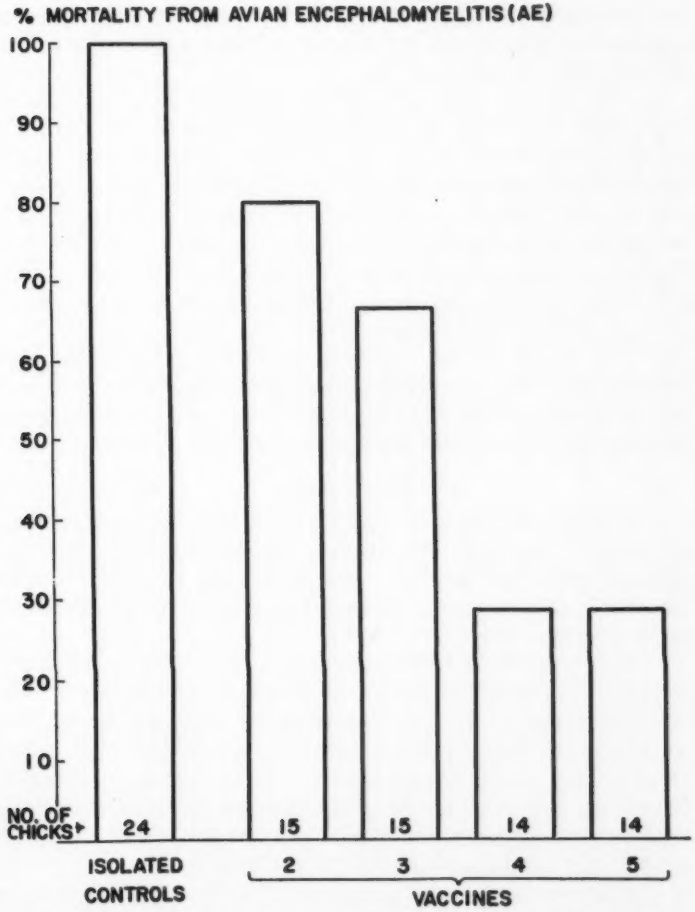

Fig. 2. Mortality from avian encephalomyelitis following intracerebral challenge among controls and among groups of chickens vaccinated intramuscularly with 1.0 ml doses of four of the BPL-inactivated AE vaccines described in table 1. The superiority of vaccines 4 and 5 is associated with the inclusion of aluminum hydroxide.

had an alkaline pH gave somewhat better protection than vaccine 2 although the apparent difference was small.

The fact that the vaccines containing aluminum hydroxide

but differing in pH were equal to one another in protecting against challenge is further evidence that differences in pH were relatively unimportant.

The more promising vaccines, numbers 4 and 5, containing aluminum hydroxide were again compared after storage in the refrigerator at 4C for 14 months (figure 3). Antigenicity was unimpaired by this prolonged storage. Although the results seem to favor vaccines with an alkaline pH, it should be noted that the difference in mortality between vaccinated chickens and their respective controls in pens 1 and 2 is about the same, again indicating that there was probably no real difference in antigenicity.

The results of these tests showed that inactivated virus vaccines induced a high degree of protection from AE during a period of at least 5 to 7 weeks, and retained their potency throughout the prolonged period of storage.

DISCUSSION

It is interesting to note in figure 1 that the smallest dose of vaccine employed provided protection against challenge equal to that produced by a dose five times as large. Similar observations were made several times in titration challenge tests not reported here.

This appears to be an example in chickens of a phenomenon previously observed by Schlesinger[7], in studies of the viruses of equine encephalomyelitis in mice. In citing this work, Burnet[1] states: "Schlesinger . . . has shown that, when virus is inoculated intracerebrally in the immune animal, a large dose may act as a 'booster' stimulus to rapid secondary type antibody production in the local region. Particularly if a relatively slowly multiplying strain is used, this may result in the paradoxical result that some lightly immunized mice receiving large challenge inocula may survive while those receiving smaller inocula succumb". Since the incubation period of AE in a completely susceptible young chicken is rarely less than nine days, even when injected intracerebrally, the AE virus can be considered to fit into the category of a relatively slowly multiplying virus.

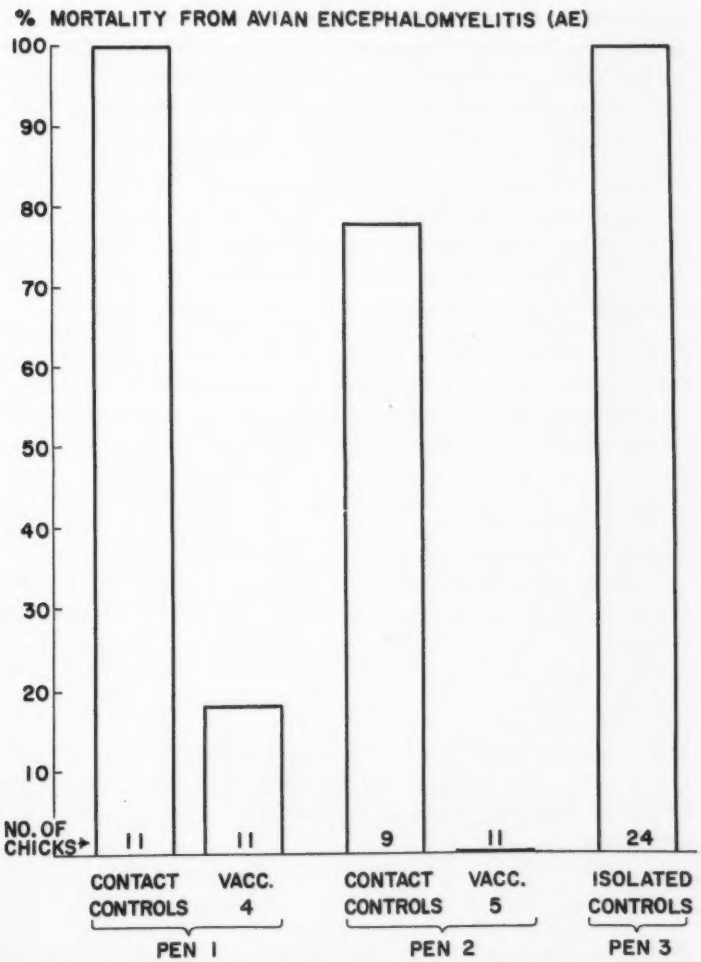

Fig. 3. Mortality from avian encephalomyelitis following intracerebral challenge among controls and among groups of chickens vac-intramuscularly with 1.0 ml doses of vaccines 4 and 5 (see table 1) after storage at 4 C for 14 months.

A vaccination dose of inactivated virus as small as 0.1 ml (figure 1) should not be considered adequate protection against field exposure, however, since the achievement of this apparent phenomenal resistance may require a large dose of the virus injected directly into the brain. It is unlikely that the level of exposure and route of infection normally encountered in natural outbreaks will parallel the experimental procedure cited. Furthermore, the work of Jungherr and Minard[2] has suggested that the natural route of infection for AE, other than that of egg transmission, is by way of the alimentary tract.

An intracerebral challenge dose which is sufficient to produce symptoms in all or nearly all of the control chickens has been used in this study because a simpler test of immunity, employing usual serological procedures, is lacking. Under these rigorous conditions of challenge, the survival of 80 percent or more of the vaccinated chickens is evidence of the production of a very high degree of useful protection and survival of 50 percent is believed to represent a degree of immunity more than adequate to offset natural exposure.

While the inactivated virus content of the dosages employed in these trials may seem to be inordinately large, the question of their feasibility is a matter of economics. Vaccine 5, which appeared to be the most effective after prolonged storage, contained 12.5 percent brain tissue or 125 mg per dose. At this rate of dilution, the brain of one expendable cockerel chick when suitably infected with the virus of AE, would immunize about 12 laying hens. Determination of the minimum effective dose, of course, will require further experimentation.

In discussing this problem Burnet[1] suggests that it would seem reasonable to ask that about the same amount of killed virus be used as would be produced in the body during a subclinical infection, and that the killed virus be made available to the antibody-producing mechanisms over a period of time approximating the course of the natural disease. A large dose of inactivated AE virus combined with aluminum hydroxide as an adjuvant, as has been used in these tests, appears to be an effective method of approaching this basic requirement.

Of the vaccine trials reported here, the most promise appears to lie in a vaccine similar to number 5 (figure 3) containing aluminum hydroxide as an adjuvant. This vaccine gave the best protection against challenge although it contained the lowest level of inactivated virus-infected brain tissue.

ACKNOWLEDGMENTS

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Cutter Laboratories, Berkeley, California, generously furnished the 25 percent aluminum hydroxide suspension for an adjuvant.

SUMMARY

When vaccination of immature breeding stock for avian encephalomyelitis (AE), or epidemic tremor, introduces the risk of exposing susceptible breeding hens to live virus, an effective inactivated vaccine is needed. The preparation of five experimental inactivated AE vaccines is described and their effectiveness in immature White Leghorn chickens is reviewed. Beta-propiolactone was used to inactivate avian encephalomyelitis virus as harvested in brain tissue from chicks showing active symptoms of the disease following intracerebral inoculation with 35th brain passage material. Vaccines consisted of different proportions of inactivated virus alone and with aluminum hydroxide added as an adjuvant. The pH of the vaccines ranged from 4.4 to 7.4. Vaccines were administered intramuscularly in 0.1 to 1.0 ml doses containing 12.5 to 25 percent brain tissue.

Each of the vaccines which contained the aluminum hydroxide adjuvant effectively induced immunity, as shown by protection, complete in one instance, from intracerebral challenge with live AE virus five to seven weeks after vaccination. The vaccines were well tolerated by the chickens.

The best protection was obtained with a vaccine having a pH of 7.4 and containing 6.25 percent aluminum hydroxide and 12.5 percent brain tissue. This vaccine did not lose its effectiveness after storage at 4C. for fourteen months.

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SURVIVAL OF PPLO INJECTED INTO EGGS PREVIOUSLY DIPPED IN ANTIBIOTIC SOLUTIONS*

By R. R. CHALQUEST and JULIUS FABRICANT

Department of Pathology and Bacteriology
New York State Veterinary College, Ithaca, N.Y.

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THERE is substantial evidence to indicate that the pleuropneumonia-like organisms (PPLO) associated with chronic respiratory disease in chickens and infectious sinusitis of turkeys are egg transmitted. The purpose of this study was to investigate the possibility of destroying these PPLO within the hatching eggs. It was felt that if a sufficient amount of antibiotic in solution could be absorbed through the shell, the PPLO might be destroyed and control of the disease might be achieved.

Extensive work has been done on washing or dipping of eggs. Several workers[8,13,14,18,19,20] have reported that hatching eggs can be washed without untoward effect on hatchability provided the water is warmer than the eggs. Lancaster *et al.*[15] and Gordon *et al.*[9] reported that an experimental contamination of the outside of the egg shell with *Salmonella* could be controlled by dipping these eggs into disinfectant solutions that were warmer than the eggs. However, Frank and Wright[7]

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were not successful in destroying *S. typhi-murium* with a similar dipping procedure.

A few workers[3,17,21] have attempted to increase the storage life of eggs dipped in antibiotics without success. Elliott and Romoser[5] dipped eggs into antibiotic solutions that were colder than the eggs. They found assayable quantities of the antibiotic in these eggs well above the in vitro levels necessary to kill many gram negative bacilli capable of producing spoilage in eggs. They contended that eggs properly dipped into antibiotic solutions may have an extended shelf life. There have been numerous reports on the use of various antibiotics to control PPLO; however, only a few workers[10,12,24,25] have tested the effect of the various drugs on this agent within the egg. In this study the effects of dipping hatching eggs into solutions of oxytetracycline, erythromycin, and streptomycin on the survival of PPLO inside the egg were noted. All the experiments were done under closely controlled conditions.

The conception of dipping hatching eggs into bactericidal liquids to control bacterial infections in or on the eggs has been patented by Seltzer.[22]

MATERIALS AND METHODS

All chicken hatching eggs used in this study were obtained from the Cornell poultry disease research farm flock. There was no evidence of any respiratory infection in this flock during this study. Repeated attempts to isolate PPLO from these birds or their eggs have always failed. Periodic agglutination tests of this flock have never resulted in any positive reactions for PPLO. All turkey hatching eggs used in this study were obtained from the College of Agriculture turkey farm. Both Empire White and Broad Breasted Bronze eggs were produced by birds which were not known to have undergone any respiratory disease.

Before dipping, the hatching eggs were warmed to 37C in a commercial, forced draft, egg incubator for 3 hours on egg flats as indicated by Law and Kosin.[16] The antibiotic solutions in galvanized metal bushel containers were prepared and cooled to the proper temperature in a refrigerator 3 hours

prior to dipping of the eggs. The solutions, made with tap water, were thoroughly agitated by an electric propeller type mixer. Two different detergents, Triton† NP-56 (nonyl phenoxypolyethoxy ethanol) and Triton X-100 (iso-octyl phenoxypolyethoxy ethanol), were used in this study. Both of them were liquid, anhydrous, non-ionic, surface active agents. Either Triton NP-56 was used at a concentration of 0.1 percent or Triton X-100 was used at a concentration of 0.05 percent in the dip solution. The actual forms of the three antibiotics used in this study were as follows: Oxytetracycline (OTC) which was used as "Terramycin Poultry Formula, Liquid Concentrate,"* contained 25 mg of the active ingredient per milliliter. Erythromycin (ERY) was used as "Gallimycin Poultry Formula,"** a powder, which contained 25 grams of the active ingredient per pound. Streptomycin (Strep) was used as streptomycin sulfate. This powdered product assayed 795 gamma per mg of the active ingredient and was added to the solutions on this basis.

An egg basket containing about 100 warmed eggs was lowered into 15 liters of dip solution so that all the eggs were completely immersed. The mixer was used to circulate the dip solution. About half way through the dipping time, the eggs were gently shifted in position while still in the circulating dip solution to insure exposure of the entire shell. At the end of the dipping time, the eggs were dried in the egg basket with the aid of a small electric fan. All eggs were incubated in commercial, forced draft incubators and were turned three times each day. All the chicken hatching eggs were transferred to the hatchers after 18 days of incubation and the turkey eggs were transferred after either 21 or 24 days of incubation. All eggs believed to be infertile by the candling process when they were being transferred to the hatcher were broken and examined grossly for evidence of embryo development. Any egg with signs of embryo development was classified as fertile.

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† Rhom and Haas.
* Chas. Pfizer and Co.
** Abbott Laboratories

PPLO strain 293 was used throughout this study. This particular strain was chosen because it was a pathogenic S_6 type and many of its growth characteristics and pathogenic properties had been determined by Calnek[2] and Fabricant.[6] It was relatively easier to reisolate on artificial media than many of the other pathogenic strains available to us.

To introduce PPLO into the fresh eggs, a small hole was drilled through the shell just above the air cell but not through the shell membrane. With the eggs held upright in a rack, the inoculum (usually 0.1 ml) was injected into the yolk of the egg with a one ml tuberculin syringe and a 1½ inch 26 gauge needle. The needle was then withdrawn and the hole in the shell sealed with wax.

The media for cultivation of PPLO included Adler's overlay media, modified Grumbles media,[23] and Bacto PPLO agar (Difco) containing 1,000 units per ml of penicillin, thallous acetate (1:2,000 concentration), 10 percent horse serum, and one percent yeast hydrolysate (Nutritional Biochem Co.).

RESULTS

Effect of the dipping process on hatchability. Numerous experiments which included 75 trials with a total of over 6,500 chicken hatching eggs were conducted to study the effect on hatchability of completely immersing fresh, uninoculated hatching eggs at 37 C into cold antibiotic solutions at 5, 10, or 17 C. Immersion times of 30, 45, 60, 90, 120, or 180 minutes were tried. The concentrations of antibiotics tested were 100, 500, and 1,000 parts per million (ppm) of OTC and Strep and 80, 400, and 800 ppm of the ERY. Some trials were also conducted with tap water and detergent without the antibiotics. Seven trials with a total of 700 turkey hatching eggs at 37 C were immersed into 10 C antibiotic solutions. The concentrations of the solutions were 100 ppm of OTC or Strep and 80 ppm of ERY. These eggs were immersed for 30 minutes. The results of these trials with both chicken and turkey hatching eggs did not indicate any difference in the hatchability of the dipped and their undipped control eggs.

Effect of the dipping treatment on the survival of PPLO in inoculated eggs. Three experiments, B 4, B 5, and B 6, (tables 1, 2, and 3) were conducted with chicken hatching eggs. The eggs were dipped for 30 minutes in solutions containing either 500 ppm of OTC or 400 ppm of ERY. The temperature of the eggs was 37 C and that of the dip solution was 5 C. Within 4 hours after being dipped, all eggs to be infected were injected with an inoculum of 0.1 ml into the yolk sac as already described.

TABLE I

Results of reisolation attempts (experiment B 4) of PPLO from eggs previously warmed to 37C and dipped into 500 ppm oxytetracycline (OTC) or 400 ppm erythromycin (ERY). The antibiotic solutions were kept at 5 C and the immersion time was 30 minutes. All eggs were cultured at hatching time.

PPLO inoculum	Dip	Fertile Eggs	Chicks hatched	Reisolations of PPLO			
				Live chicks	Pips	D.I.S.*	Total
None	None	44	30	0/3†	0/2	0/3	0/8
None	OTC	43	38	0/3	0/1	0/2	0/6
None	ERY	35	31	0/3	0/0	0/0	0/3
N. Broth	None	40	28	0/3	0/2	0/3	0/8
10^{-3}	None	38	16	3/3	0/0	3/3	6/6
10^{-4}	None	39	17	4/4	0/0	2/3	6/7
10^{-5}	None	35	14	3/3	2/2	3/3	8/8
10^{-6}	None	36	28	0/3	0/0	2/3	2/6
10^{-7}	None	35	19	3/3	2/2	2/3	7/8
10^{-8}	None	37	30	0/3	0/1	1/3	1/7
10^{-3}	OTC	35	24	1/3	1/1	2/2	4/6
10^{-4}	OTC	31	20	3/3	0/0	3/3	6/6
10^{-5}	OTC	29	16	2/3	2/2	3/3	7/8
10^{-6}	OTC	37	24	1/3	0/0	0/3	1/6
10^{-7}	OTC	44	33	0/3	0/1	0/3	0/7
10^{-8}	OTC	41	31	0/3	0/1	0/3	0/7
10^{-3}	ERY	30	16	0/3	1/1	0/3	1/7
10^{-4}	ERY	35	24	0/4	0/0	0/2	0/6
10^{-5}	ERY	26	16	0/3	0/0	0/3	0/6
10^{-6}	ERY	30	18	0/3	0/1	0/3	0/7
10^{-7}	ERY	36	28	0/3	0/1	0/3	0/7
10^{-8}	ERY	40	24	0/3	0/2	0/3	0/8 §

* D.I.S.—Dead in shell.

† Number reisolations/Number attempts

§ The reduction in the number of isolations of PPLO from the ERY treated groups as compared with that from the non-treated control group was significant at the 0.5 percent level.

Ten fold dilutions, 10^{-3} to 10^{-8}, were made of a 48 hour broth culture with sterile nutrient broth. Each dilution was inoculated into 50 treated and 50 untreated eggs, starting with the 10^{-8} dilution and continuing with the next lower dilutions. After inoculations, the eggs were placed into a commercial, forced draft, incubator and candled on the 5th, 13, and 18th days of incubation. All dead embryos were removed at each candling and some of them were cultured for PPLO on the 13th and 18th days. When available, the yolks of 5 dead in shell embryos were inoculated into tubes of modified Grumbles' media and incubated for 14 days.

At hatching time, all live chicks and pipped embryos were examined for air sac lesions. Live chicks, pipped, and dead in shell embryos from each group were cultured when possible. In experiment B 4, three live chicks, two pips, and three dead in shell, when available, were cultured from each group. In experiments B 5 and B 6, five live chicks, two pips, and three dead in shell were cultured. However, in these last two experiments, ten reisolation attempts were always made. Live chicks were substituted when there were insufficient pipped or dead in shell embryos.

The yolk material or lesion, when present, from all chicks or embryos cultured was inoculated with a loop or sterile swab into both a tube of Adler's overlay medium and a tube of modified Grumbles' medium. The tube of modified Grumbles medium was incubated for 14 days. Two blind passages in Adler's overlay media were made from the tube of Adler's at three day intervals. A loopful from the last blind passage was transferred to a tube of modified Grumbles' medium and an additional loopful was streaked onto a plate of the solid media. The plates were allowed to incubate for six days and the Grumbles' medium for 14 days before final readings were made.

The production of acid in any of the tubes of modified Grumbles' media in which no bacteria could be demonstrated either by Gram stain or by aerobic culture on horse blood agar plates, constituted a positive isolation of PPLO. However, the majority of such isolations in experiment B 4 and all such

isolations obtained in B 5 and B 6 were also checked for growth of typical colonies for this particular strain of PPLO on the solid media. If a reisolation was obtained in the modified Grumbles' media before the completion of the blind passage in Adler's, the blind passages were discontinued for that particular reisolation attempt. If the blind passages were carried to completion, then either the growth of typical colonies for this particular strain on the solid media or the production of acid in the modified Grumbles' media in the absence of other bacteria constituted a positive isolation.

TABLE II

Results of reisolation attempts (experiment B 5) of PPLO from eggs previously warmed to 37 C and dipped into 500 ppm oxytetracycline (OTC) or 400 ppm erythromycin (ERY). The antibiotic solutions were kept at 5 C and the immersion time was 30 minutes. All eggs were cultured at hatching time.

PPLO inoculum	Dip	Fertile Eggs	Chicks hatched	Reisolations of PPLO			
				Live chicks	Pips	D.I.S.*	Total
None	None	43	34	0/6†	0/1	0/3	0/10
None	OTC	44	35	0/7	0/0	0/3	0/10
None	ERY	42	32	0/6	0/2	0/2	0/10
N. Broth	None	33	21	0/5	0/2	0/3	0/10
10^{-3}	None	31	10	9/9	0/0	1/1	10/10
10^{-4}	None	33	11	6/6	1/2	3/3	10/11
10^{-5}	None	28	16	6/6	2/2	2/2	10/10
10^{-6}	None	34	23	6/6	2/2	2/2	10/10
10^{-7}	None	30	17	5/7	0/0	1/3	6/10
10^{-8}	None	35	21	7/7	0/0	2/3	9/10
10^{-3}	OTC	31	19	7/7	2/2	1/1	10/10
10^{-4}	OTC	34	12	5/5	2/2	3/3	10/10
10^{-5}	OTC	37	22	5/5	2/2	3/3	10/10
10^{-6}	OTC	31	20	6/7	0/0	2/3	8/10
10^{-7}	OTC	32	22	5/7	0/0	1/3	6/10
10^{-8}	OTC	33	22	5/7	0/0	0/3	5/10
10^{-3}	ERY	39	22	5/5	2/2	3/3	10/10
10^{-4}	ERY	36	18	5/5	2/2	0/3	7/10
10^{-5}	ERY	31	23	1/5	2/2	0/1	3/8
10^{-6}	ERY	38	31	2/8	0/0	0/2	2/10
10^{-7}	ERY	34	23	1/7	0/0	0/3	1/10
10^{-8}	ERY	39	29	2/6	0/1	0/3	2/10§

* D.I.S.—Dead in shell.

† Number reisolations/Number attempts.

§ The reduction in the number of isolations of PPLO from the ERY treated groups as compared with that from the non-treated control group was significant at the 0.5 percent level.

In experiment B 4, no air sac lesions were observed in any of the live chicks or pipped embryos at hatching time. Many reisolations of PPLO were obtained by hatching time as indicated in table 1. Some additional reisolations of PPLO were made from the dead embryos cultured on the 13th and 18th days of incubation. Therefore, these deaths are thought to be due to the PPLO which were inoculated. The differences in the number of reisolations of PPLO at hatching time in the ERY treated and the control groups inoculated with the various dilutions were statistically significant at the 0.5 percent level by the Chi square test. There were no significant differences between the OTC treated groups and the controls.

In experiment B 5, caseous air sac lesions were observed in some of the PPLO inoculated but undipped groups. Two live chicks from the group inoculated with the 10^{-3} dilution, two live chicks in the 10^{-4} dilution, and four live chicks in the 10^{-5} dilution group had caseous air sac lesions. No lesions were observed in any of the groups that had been dipped or in the uninoculated controls. Reisolations of PPLO were obtained in most of the groups as indicated in table 2. Although more reisolations were obtained in the dipped groups in this experiment than were obtained in the previous trial, the ERY dipped eggs still yielded significantly fewer isolations than the undipped inoculated groups at the 0.5 percent level. The OTC dipping did not yield significantly different results from the controls.

In experiment B6, caseous air sac lesions were observed in some of the undipped, inoculated groups and also in one group that had been dipped in OTC and inoculated. In the undipped but inoculated groups, four live chicks in the 10^{-3} dilution group, two live chicks in the 10^{-4} dilution group, and one live chick in the 10^{-7} dilution group had caseous air sac lesions. In the OTC dipped groups one chick from the 10^{-4} dilution group had caseous air sac lesions. Reisolations of PPLO were obtained in many of the groups as indicated in table 3. The Chi square analysis in the experiment indicated a significant difference in the number of reisolations obtained in both the OTC dipped groups and the ERY dipped groups

from the undipped inoculated groups at the 0.5 percent level.

TABLE III

Results of reisolation attempts (experiment B 6) of PPLO from eggs previously warmed to 37 C and dipped into 500 ppm oxytetracycline (OTC) or 400 ppm erythromycin (ERY). The antibiotic solutions were kept at 5 C and the immersion time was 30 minutes. All eggs were cultured at hatching time.

PPLO inoculum	Dip	Fertile Eggs	Chicks hatched	Reisolations of PPLO			
				Live chicks	Pips	D.I.S.*	Total
None	None	41	35	0/8†	0/1	0/1	0/10
None	OTC	41	34	0/10	0/0	0/0	0/10
None	ERY	40	31	0/6	0/2	0/2	0/10
N. Broth	None	35	23	0/6	0/1	0/3	0/10
10^{-3}	None	32	16	5/5	2/2	3/3	10/10
10^{-4}	None	22	15	6/6	1/1	3/3	10/10
10^{-5}	None	35	25	5/5	2/2	3/3	10/10
10^{-6}	None	26	17	6/7	0/0	3/3	9/10
10^{-7}	None	25	21	8/9	0/0	0/1	8/10
10^{-8}	None	24	18	6/7	0/0	1/3	7/10
10^{-3}	OTC	25	6	5/5	2/2	2/3	9/10
10^{-4}	OTC	30	19	6/6	1/1	1/3	8/10
10^{-5}	OTC	30	20	3/6	0/1	0/3	3/10
10^{-6}	OTC	26	15	0/5	0/2	0/3	0/10
10^{-7}	OTC	28	22	1/8	0/0	0/2	1/10
10^{-8}	OTC	29	13	0/7	0/0	0/3	0/10§
10^{-3}	ERY	32	15	3/5	0/2	2/3	5/10
10^{-4}	ERY	28	19	0/5	0/2	0/3	0/10
10^{-5}	ERY	34	25	0/6	0/1	0/3	0/10
10^{-6}	ERY	33	20	0/5	0/2	0/3	0/10
10^{-7}	ERY	22	13	0/6	0/1	0/3	0/10
10^{-8}	ERY	29	16	0/8	0/0	0/2	0/10§

* D.I.S.—Dead in shell.
† Number reisolations/Number attempts.
§ The reduction in the number of isolations of PPLO from the OTC and the ERY groups as compared with that from the non-treated control group was significant at the 0.5 percent level.

The number of reisolations obtained in the undipped inoculated groups of these three experiments are very similar. From the data of the undipped inoculated groups, the ELD_{50} for experiment B 4 was $10^{-5.0}$, that of experiment B 5 was $10^{-4.36}$, and that of experiment B 6 was greater than 10^{-3}. However, experiments B 4 and B 5, which had almost identical inoculums based on the calcuated ELD_{50}, yielded markedly different results in the actual numbers of reisolations of PPLO at hatching time. Further work is necessary to deter-

mine the reason for the variations in the reisolations of PPLO in these experiments.

Assays of antibiotics obtained from undipped and dipped eggs. Assays of oxytetracycline in yolks and albumens from dipped and undipped eggs were conducted by Mr. D. C. Billman of Chas. Pfizer and Co., Inc., Terre Haute, Indiana, by means of the standard cylinder-plate technique with *Bacillus cereus var. mycoides* as described by Grove and Randall[11]. Twenty-four assays were done on composite samples of the yolk or albumen from three eggs per sample as suggested by Elliott[4]. Three assays were done for each concentration of dip solution. The eggs previously incubated at 37 C for 3 hours had been dipped in an oxytetracycline solution at 5 C for 30 minutes. The concentration of the dip solution was 100, 500, or 1,000 ppm. A control group of undipped eggs were checked in a similar manner. The quantities detected in the yolks ranged from less than 0.15 to 0.50 micrograms per gram. The quantities present in the albumens ranged from 0.405 to 7.50 micrograms per gram.

Although the amounts of OTC found in the albumens were not directly proportional to the concentration in the dip, there was a definite increase in the eggs dipped in each higher concentration of antibiotic. For some unknown reason, concentrations of OTC in yolks of eggs dipped in 500 ppm solution were definitely higher than the values in yolks of eggs dipped in 1,000 ppm.

DISCUSSION

The efficiency of the dipping procedure described in this paper with respect to its effect on hatchability is probably due to the fact that very little, if any, bacterial contamination occurred during the dipping and to the fact that the levels of antibiotics used in this study were not toxic. The major sources of bacterial contamination of such a procedure are the dip solution itself and any contaminated material present on the outside of the egg shell. Contamination by the dip solution or any material on the outside of the egg shell

was probably prevented by the relatively high, 80 to 1,000 ppm, concentrations of the antibiotics used and to the 3 hours holding period at 5 C prior to the use of these dipping solutions. Whether similar results would have been obtained with shorter holding period after the addition of the antibiotic was not investigated in this work.

A series of experiments were conducted to test the effect of dipping on the survival of PPLO inoculated into fresh hatching eggs. Based on the number of reisolations of strain 293 PPLO obtained at hatching time and within the limits of the experimental procedure, the dipping process appeared to be a very promising method of killing PPLO. In three experiments, B 4, B 5, and B 6, the inoculated eggs that had been dipped into erythromycin yielded significantly fewer reisolations of PPLO than were obtained from the undipped inoculated groups. However, more reisolations of PPLO were obtained in one of these trials, experiment B 5, than the others. The reason for the increased number of reisolations in this particular trial is unknown.

A source of variation is the reisolation technique itself. The technique used for the reisolations of PPLO is thought to be the most efficient one available at this time. However, there is little question, as indicated by Fabricant[6], that it is not entirely satisfactory. It is possible that PPLO were actually present although no reisolation could be made. An exact interpretation of these results is not possible until the technical problems of working with these organisms are solved.

The difference in the effect produced by the two antibiotics might be explained in at least three different ways. First, strain 293 may be more sensitive to ERY than it is to OTC. The relative sensitivity of this strain to these two antibiotics is unknown at this time. Next, OTC and ERY may differ in their ability to penetrate the egg shell. OTC is known to have some affinity for calcium ions, and it may be that too much of this drug is bound in the shell to allow the proper concentration inside the egg itself. Also, there may be a dif-

ference in the stability of these drugs in incubating eggs. Because of these factors, it is felt that additional work is necessary with these two drugs as well as with others. Since only one strain of PPLO was used in this entire study, more work is also required to study the effect of these and other drugs on several strains of this agent.

Another important question that needs investigation is how closely does this experimental procedure correspond to natural egg transmission. Under the present limitations of techniques, it is nearly impossible to tell how many PPLO are present in a naturally infected fresh or incubated egg. The exact amounts of inoculum used in these trials may not have been equivalent to those present in naturally infected eggs. It is hard to conceive that the inoculum in a naturally infected egg is more concentrated than a 10^{-3} dilution of a 48 hour broth culture. The hatchability of the undipped groups that received this level of inoculum was markedly reduced from the undipped, uninoculated control groups. However, the amount of inoculum in a naturally infected egg may be less than a 10^{-8} dilution of a 48 hour broth culture.

The reduction in the number of reisolations of PPLO obtained, especially in experiments B 4 and B 6, suggests that the dipping of hatching eggs in antibiotics may have practical significance. The treatment is certainly simple enough to be used by any farmer or hatcheryman with very little equipment. Since the eggs used in these trials were experimentally infected and not naturally infected, more work is necessary to determine if this process will actually prevent the appearance or reduce the incidence of the disease in chicks and poults hatched from naturally infected eggs. However, the rate of egg transmission in a natural infection is usually so low and so irregular that proper evaluation of this dipping process on such material would have to be made from many experiments on infected flocks over an extended length of time.

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SUMMARY

1. This study was conducted to evaluate a possible means of killing PPLO in eggs. The method consisted of dipping warm hatching eggs into cold antibiotic solutions in an attempt to produce sufficient absorption of the antibiotic from the dip solution into the egg to prevent infection of the resulting chick or poult.

2. Oxytetracycline assays of some yolks and albumens were obtained from some dipped and some undipped eggs. The dipped eggs had been warmed to 37 C and dipped in an oxytetracycline solution at 5 C for 30 minutes. The concentration of the dip solution was 100, 500, or 1,000 ppm. The quantities detected in the yolks ranged from less than 0.15 to 0.50 micrograms per gram. The quantities present in the albumens ranged from 0.405 to 7.50 micrograms per gram. Apparently, the antibiotic from the dip solution does enter both the albumen and the yolk of the egg during the dipping process.

3. The effect on hatchability of dipping warm hatching eggs into cold antibiotic solutions was studied. Seventy-five trials with a total of over 6,500 chicken hatching eggs and 7 trials with a total of 700 turkey hatching eggs were used in this part of the study. Chicken hatching eggs at a temperature of 37 C were completely immersed in antibiotic solutions at

5, 10, or 17 C for periods of 30, 45, 60, 90, 120, or 180 minutes. Turkey hatching eggs also were immersed in varied concentrations of antibiotic solutions and there appeared to be no difference in the hatchability of the dipped eggs and their respective undipped controls under the conditions studied.

4. To test the effect of the dipping process on the survival of PPLO in eggs, fresh chicken hatching eggs were inoculated with 0.1 ml of a dilution (10^{-3} to 10^{-8}) of a 48 hour broth culture of PPLO. Strain 293, an S_6 pathogenic type PPLO, was used as the infective agent throughout this study. Two groups of these eggs at a temperature of 37 C had been previously dipped for 30 minutes either in 400 ppm erythromycin solution or in 500 ppm oxytetracycline solution held at 5 C respectively. The third and fourth groups of eggs served as controls. Reisolations of PPLO were attempted at hatching time from live chicks and pipped and dead in shell embryos. In all three trials there were significantly fewer reisolations obtained from the inoculated groups that had been dipped in the erythromycin solution than were obtained from the undipped inoculated groups. In two of these trials, experiments B 4 and B 6, reisolations of PPLO were not obtained from any of the chicks or embryos originating from eggs inoculated with dilutions higher than 10^{-3}. The eggs that had been dipped into the oxytetracycline solution yielded significantly fewer reisolations of PPLO in one of the three trials. Inhibition in this case was observed in eggs inoculated with dilutions of PPLO culture 10^{-5} or greater.

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STAINING FIXED PREPARATIONS OF CHICKEN BLOOD CELLS WITH COMBINATION MAY-GREENWALD-WRIGHT-PHLOXINE B STAIN*

By F. W. COOK

Department of Poultry Science
North Carolina State College
Raleigh, North Carolina

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AMONG the stains and stain combinations employed in staining fixed blood preparations, the most common are May-Greenwald, Giemsa, Wright-Giemsa and May-Greenwald-Giemsa. The technics in this paper were designed to produce a spreading method which would make uniformly thin smears of chicken blood with the cells evenly distributed and to have a stain and staining procedure which would differentiate more specifically the several types of chicken blood cells, particularly lymphocytes and monocytes. These procedures are presented here with the purpose of aiding others using avian blood in research and in the clinic.

MAKING THE BLOOD FILMS

Materials needed for making blood films

Precision-grade microscope slides (0.8-1.0 mm)
Crayon for waxing slides
Applicator for transferring blood
Glass blocks for spreading blood
Knife blades
Methyl alcohol

* Contribution No. 985 of the North Carolina Agricultural Experiment Station.

Anticoagulant solution
Silicone solution
Detergent solution
Acid cleaning solution

(a). The glass spreader* (figure 1) is approximately 1 x ⅞ x ¼ inches in size. The spreading area has a finely ground surface.

(b). The applicator (figure 1a) for transferring the blood may be made from a strong wire stylet of 16 or 17 gauge wire, 3 or 3½ inches in length. The bevelled end of the stylet is snipped off and the cut end of the wire is heated and shaped to form a round loop approximately 2 mm in diameter. The shaft is bent with the aid of heat to form an angle, near the loop, of approximately 90 degrees. The cut end of the wire forming the loop is placed against the shaft and held in position with liquid solder.

(c). The anticoagulant solution for cleansing the skin at the site of the puncture for the blood and for rinsing the wire loop after each transfer of blood to the slide is composed of:

Sodium citrate, USP.	$Na_3C_6H_5O_7.2H_2O$	3.0 gm
Sodium chloride, NaCl.		1.0 gm
Distilled water to make		100.0 ml

(d). The silicone solution† may be used to coat the loop of the applicator as an aid in retarding coagulation of the blood while transferring it to the slide.

(e). Any brand of detergent dissolved in tap water may be used for holding and washing the glass blocks to remove the blood after spreading. Before re-use the blocks are rinsed, in sequence, with tap water and methyl alcohol, and then dried by placing each block on its side on a paper towel to permit the evaporation of the alcohol. Periodically the blocks are chemically cleaned with dichromate-sulfuric acid solution.

(f). The slides are waxed by making 8 to 10 parallel lines lengthwise on one side with a glass-marking wax crayon

* Obtained on special order from Arthur H. Thomas Company, Philadelphia, Pa.

† Dri-Film SC-87, General Electric Silicone Products Department, Waterford, N. Y.

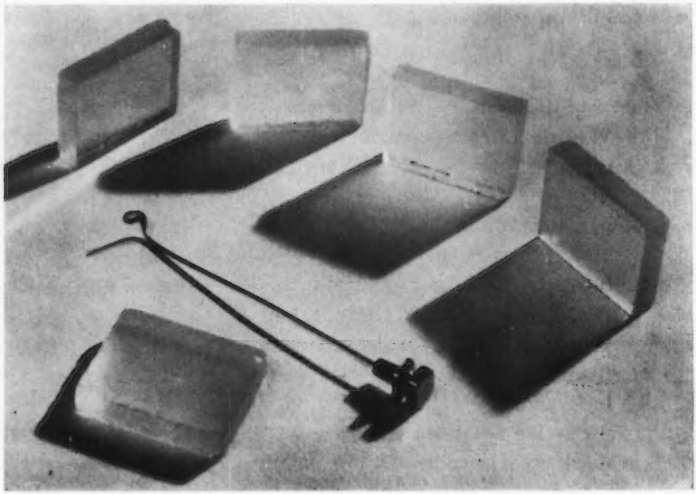


Fig. 1a. Photograph of applicator and glass blocks for transferring and making blood films.

and rubbing the wax over the entire surface of the slide with a clean cloth of firm texture. The wax should have a high melting point to remain hard during hot weather. The primary purpose in waxing and polishing the slides is to provide a smoother-than-glass surface upon which the blood can spread as rapidly and uniformly thin as possible without damage to the cells, and to obtain as even a distribution of cells as possible throughout the blood film.

Method of making the blood films.

1. The site of the puncture for the blood is wiped clean with the anticoagulant to remove debris and to coat the area around the puncture with anticoagulant to retard coagulation of the blood on contact with the skin.

2. A wing vessel is incised with a sharp-pointed blade. The first drop of blood is removed with a cloth to obtain blood free of tissue juice from the incision.

3. The loop of the applicator is immersed in the blood and speedily transferred onto one end of a waxed slide. The volume of blood placed on the slide may be limited quite

accurately by restricting the area of the loop applied to the slide. The loop of the applicator should be rinsed in the anticoagulant immediately after use and air dried.

4. The spreader, long axis parallel to the end of the slide, is held between the thumb and index finger and quickly placed on the blood. When the blood has spread almost to its greatest area under the block, the spreader is pulled quickly and steadily to the opposite end without applying downward pressure.

5. The blood is dried quickly by waving the slide in the air.

6. The glass block is placed in a dish of detergent solution to hold until ready for re-use.

The essentials of this spreading technic are: (1) waxed slides free of dust, (2) spreaders free of dried blood on spreading surface, (3) blood fresh from a vessel for each preparation, that is, not to be taken from a long standing pool of blood, (4) blood free of debris and tissue juice from the incised skin and vessel, (5) speed in transferring the blood on the loop of the applicator to the slide, (6) sufficient volume of blood on the slide to spread almost to the boundaries of the block, (7) pulling the spreader quickly and steadily without downward pressure, and (8) drying of the blood film.

The blood cells will stain equally well when spread by the 2-slide method used in some laboratories, however, it is the belief of the writer that this spreading procedure has the following advantages: (1) the drop of blood placed on the slide can be limited in size as desired, (2) the blood films can generally be made uniformly thin with a minimum or no "piling up" of cells at the sides of the smear, (3) the combination of the ground surface of the spreader and the waxed surface of the slide forces the blood to spread in all directions under the block exhibiting in most instances, with the microscope, an even distribution of the cells over the entire surface of the blood film, (4) the weight of the block appears to make the cells extend to their maximum surface diameters and minimum thickness thus assuring maximum flattening

of the cells for wider surface distribution and clearer visibility of their stained inclusions, thereby enhancing their finer structural characteristics, color definition and differentiation consequently allowing a more accurate qualitative study of the stained cells and interpretation of the blood picture.

STAINING THE BLOOD FILMS

Materials and methods for preparing stain and buffer solutions

Stock stain

May-Greenwald (dry powder) National	2.5 gm
Wright (dry powder) National, certified	2.0 gm
Phloxine B (dry powder) certified for use in histology	0.5 gm
Methyl alcohol, absolute (acetone-free), certified A.C.S.	1.0 liter

The dry powders are added to the methyl alcohol and put into solution by vigorous and frequent shaking the first 6 to 12 hours and intermittently thereafter until filtered. This stain remains stable, unfiltered or filtered, for weeks at room temperature.

Buffer solutions

A. *Stock M/6 sodium phosphate buffer solution.*

Make fresh for each day's supply

Sodium phosphate, Na_2HPO_4, secondary, anhydrous salt	2.367 gm
Distilled water to make	100.0 ml

B. *Stock M/5 potassium phosphate buffer solution.*

Make fresh for each day's supply

Potassium phosphate, KH_2PO_4, primary, Sorenson salt	2.722 gm
Distilled water to make	100.0 ml

It is convenient to keep weighed salts (A and B) in separate vials for immediate use.

Solutions A and B may be kept in separate containers to be available for varying the pH of the buffer mixture (C) and buffered water (D) whenever necessary to modify the staining reactions produced by the diluted stain.

C. *Stock buffer mixture.*

Make fresh for each day's supply

Stock buffer solution (A and B)	Equal volumes

D. *Buffered distilled water.*

Make fresh for each day's supply

Stock buffer mixture (C)	2.0 ml
Distilled water to make	100.0 ml

The above buffer solutions are made fresh for each day's supply to eliminate the possibility of the addition of another variable in the staining procedure. If any change has occurred in the staining characteristics of the components of the cells, the question naturally arises whether the variations are due to a change in the chemistry, morphology of the cells themselves or both due to a change in the pH of the buffer solutions produced on standing in their containers for undetermined periods of time.

The pH readings of the stain and solutions:

Distilled water	6.52
M/6 sodium phosphate solution (A)	8.58
M/5 potassium phosphate solution (B)	4.30
Buffer mixture (C)	6.35
Buffered water (D)	6.71
Stock liquid stain	7.65
Methyl alcohol, absolute, acetone-free A.C.S.	6.50
Acetone	6.25
Tap water	7.40

Diluted stains:

1 part stain plus 5 parts diluent (D)	7.1
1 part stain plus 8 parts diluent (D)	7.0
1 part stain plus 10 parts diluent (D)	7.0
1 part stain plus 12 parts diluent (D)	6.98

The pH readings were made with the Beckman glass electrode pH meter, model H2.

In order to produce the tinctorial variation and specificity in color reactions for maximum definition and differentiation of cell components and cell types, the buffer system was

purposely kept flexible to be able to create and adjust the environment in the staining solutions for optimum color reactions in the cells. The molarity of the stock buffer solutions A and B, the proportions of these solutions composing buffer mixture C, the dilution of buffer mixture C to make buffered water D were constantly altered until the colors in the cells were considered specific and of differential value.

Materials and methods for staining blood films.

Staining racks
Staining dishes
Buffer salts
Buffer solutions
Stock stain
Methyl alcohol
Acetone
Absorbent paper
Beakers, graduated cylinders

(a). Have ready for use 1 dish with full-strength stock stain, 1 dish for diluted stain, 1 dish with tap water, and 3 dishes with acetone for removing excess stain from the blood films and for drying the slides after rinsing with tap water. Have equipment free of dried stain from previous use.

(b). Have sufficient stock liquid stain (fixing agent) in dish 1 to fully cover the slides. This supply of stain may be used for a large number of slides. Stain must be discarded whenever any deterioration is noted in the quality of staining in the cells.

(c). Have ready to mix, before immersing slides (step 2), the required quantities of stock liquid stain and of diluent (buffered water D), to make diluted stain.

(d). It is advantageous to have dishes longer and wider than the tray, particularly for the diluted stain, to be able to move the tray in different directions to agitate the contents and to hasten the removal of the preceding liquid from the slides thereby permitting a more uniform, rapid staining action by the solutions on the total area of the blood films.

Step 1. Fix blood films in stock stain 15 minutes or

longer. Raise and lower tray of slides 8 to 10 times, when first put into dish, to agitate the stain.

Step 2. Prior to transferring and immersing slides in diluted stain at end of fixing (step 1), have ready in separate graduated cylinders measured quantities of stock stain and buffered water to make diluted stain of desired dilution and quantity sufficient to fill dish 2, e.g., 1 part stock stain (100 ml), plus 10 parts buffered water (1000 ml). Make diluted stain by pouring stock stain into a beaker. Immediately, and with some force, pour the diluent into the stain to thoroughly mix the two liquids. Speedily pour this diluted stain into dish 2.

Step 3. Immediately after pouring diluted stain into dish 2, quickly transfer the tray of slides from dish 1 to dish 2 to avoid passing the slides through the film of iridescent material which forms quickly on the surface of the stain. Move the tray in all directions in the dish, except up through the surface film, to agitate the stain for better staining results. Leave slides in diluted stain 15 to 30 minutes.

Step 4. Remove iridescent layer from the surface of the stain with absorbent material—facial tissues or strips of paper towel. Repeat until surface of stain is clean.

Step 5. Transfer tray to dish 3 containing tap water. Rinse slides thoroughly by raising and lowering tray 8 to 10 times.

Step 6. Transfer and rinse slides, in sequence, in the 3 dishes of acetone. Raise and lower tray 8 to 10 times in each rinse. Since stain and water are added to the acetone in dish 1 with each tray of slides this rinse should be discarded frequently. Dishes 2 and 3 should be advanced to positions 1 and 2, and dish 1 to position 3. Refill dish 3 with fresh acetone.

Step 7. Remove tray from last acetone bath and allow slides to dry.

Step 8. If necessary to remove stain on slides adjacent to blood films and back of slides use a cloth moistened with acetone.

Step 9. Inspect blood films for staining results.

The color qualities, the definitiveness of the size and shape of the stained blood cells with their inclusions were used as criteria in evaluating the stained cells. Evaluations and interpretations of the cells were made on stained smears using thin slides (0.8-1.0 mm), thinly spread films, tungsten ribbon filament, 6 volt-108 watt microscope lamp, combination of filters, apochromatic, oil immersion lens, and compensating oculars (12.5X). The combination of filters adopted as standard in this procedure consisted of Daylite filter, ground glass filter, Didymium filter (Code number, Filter 5120, Corning Glass Works, Technical Products Division, Corning, N.Y.), and a neutral tint filter when needed to decrease the intensity of the light from the lamp. The Didymium filter, in combination with the other filters, enhances the definitiveness of the components of the cells and their color patterns, accentuates the existence of red-stained inclusions and intensifies the contrasts between the colors in the stained cells.

The blood films are fixed in the alcoholic stock stain 15 minutes or longer to assure adequate staining of the cortex or shell of the preformed granules of the mature heterophils in the peripheral blood, otherwise, only the inner granules[2] will be fixed and stained showing the cytoplasm of the cell with only round, deep-red granules instead of the sharp-pointed or blunt-ended, elliptical, mature granules.

Mixing the stock liquid stain with the buffered diluent immediately before immersing the slides is required in this technic to stain the basophilic granules present in the mature basophils in the peripheral blood. Basophilic granules may be unstained in some cells in the same smear with cells containing these blue-black preformed granules.

A satisfactorily stained preparation for critical definition and color differentiation of the cell structures and cytoplasm is dependent on: sufficient dilution of the stock stain with the buffered water in step 2, thorough mixing and agitation of the diluted stain before immersion of the blood films to obtain adequate staining of the granules in the mature blood basophils, (step 2), length of staining time to control

contrast and tinctorial intensities in all stainable components of the cell, (step 3), and preventing iridescent material adhering to the blood films. With the materials in use in this laboratory, a dilution of 1 part stock stain in 10 parts buffered water acting on the blood films 20 to 25 minutes gives, in most instances, the optimum environment for specific, differential staining in the cells.

The presence of azurophilic granules or particles in the cytoplasm of the lymphocytes and monocytes is one of the most important criteria demonstrating the efficiency of this staining procedure. Custer[1] recommends that the term "azurophil" rather than the term "azur" be applied to the granules seen typically in the cytoplasm of cells of the lymphocytic and monocytic series since, as he states, the term "azurophil" refers to an affinity for a particular dye and not to the color of the granules. The number, size, shape, color and distribution of the azurophilic granules or particles in the cytoplasm of the lymphocytes and monocytes of peripheral blood of the chicken are important differentiating characteristics of these cells. The total absence of these inclusions in all these cells in the preparation indicates, in this technic, the need for an adjustment either in the concentration of the dye in the diluted stain or in the staining time, or both.

The granules and cytoplasm in the stained heterophils may show little or almost no contrast in color if the dye in the diluted stain is too concentrated, e.g., 1 part stain plus 5 parts diluent, making it difficult to distinguish the granules from the cytoplasm. The cytoplasm generally becomes very light in color or almost colorless if a diluted stain containing 1 part stain plus 10 parts of diluent is used. With this latter dilution, the color in the preformed granules is sufficiently retained to give definition to the granules when observed against the lighter colored or colorless cytoplasm.

The intensity of color in the nuclei, in the cytoplasm and its inclusions, particularly in the lymphocytes and monocytes, increases as the concentration of the dye in the diluted stain is increased. The range in dilution of the stain for controlling

the color differentiation in the blood cells is between 1 part stock stain plus 8 to 12 parts buffered diluent. The average is 1 plus 10.

If, on inspection of the stained blood smears, one type or all types of cells are understained, the entire process of staining may be repeated with the same preparation without appreciable overstaining of the cells. If the cells are considerably overstained it will be necessary to stain another blood film with a diluted stain of lower concentration of dye with the same staining time or to stain with the previous dilution with a shorter staining time.

Slides with blood films which have been stored for some time prior to staining, may be covered with a film of moisture and debris. This film covering the blood smear and slide may retain the stain even after being rinsed in the three changes of acetone. This excess stain usually can be removed by returning the slides to the third acetone bath, leaving them in the bath for 2 or 3 minutes or longer without apparent destaining of the blood cells.

Periodically a new lot of dry stain is added and mixed with the current supply of powdered stain. In this way the quality of each new lot of liquid stain will vary but little from that of the preceding supply thus assuring quite constant results. However, each new lot of stock liquid stain may give sufficient variation in quality and staining reaction to necessitate one or more modifications in the procedure such as: (1) changing the proportions of stock liquid stain and diluent, (2) increasing or decreasing the staining time in the diluted stain, and (3) changing the proportions of the buffer stock solutions when making the buffer mixture. All steps in the preparation of the stock liquid stain and buffer solutions should be checked carefully before making any of the above modifications, when using a new lot of stock liquid stain, to be positive that errors were not made coincident with their preparation. Modification 1 may be tried first, then modification 2. A combination of 1 and 2 may be required. If drastic changes are needed or desired to correct or to ac-

centuate the color intensities differentiating certain cell structures modification 3 may be used.

The color quality, optimum definition and differentiation of the structures and cytoplasm in the several blood cell types appear to be dependent on the presence of and interaction of: the chromophilic substance in the structural and amorphous components of the cells, the environment of the cells influenced by the plasma of the individual birds, the concentration of the dye in the stock liquid stain, the pH of the stock liquid stain, the concentration of the salts in the buffer mixture and buffered water, the pH of the buffered diluent, the final dye concentration and pH of the buffered, diluted stain, the pre-mixing of the stock liquid stain with the buffered diluent before immersing the blood smears in the diluted stain, the time that the stock liquid stain is in contact with the blood film, and the time that the buffered, diluted stain is in contact with the fixed film.

STAINING RESULTS

The blood films for selection of cells for the following color descriptions were from apparently normal 5 and 10-week-old chickens started and reared in laboratory batteries.

Staining of nuclei. The contrast in depth of color between the chromatin and the nucleoplasm and between nuclei of different cells is an important differential characteristic, as well as differences in chromatin pattern, distinguishing cell types, particularly monocytes and lymphocytes. With this stain, the color of the chromatin of the nuclei in the cells ranges from red-purple to black-purple depending on the cell type and maturation of the nucleus. The nucleoplasm varies in color only slightly from red-purple in most of the cell types.

Erythrocytes (figure 1). Present in all photomicrographs. The color of the cytoplasm in the mature red cell of the peripheral blood ranges from pink-red to yellow-brown depending on the individual chicken, the dilution of the stain, and on the staining time. Occasionally a dark-red granule may be observed near one end of the cell.

Fig. 1, (top row, left). Heterophil. Spindle-shaped granules well contrasted against cytoplasm.

Fig. 2, (top row, middle). Heterophil. Black granules probably are the inner granules of the preformed granules of the cell or small preformed granules stained black. Some preformed granules ovoid in shape.

Fig. 3, (top row, right). Basophil. A few basophilic granules show reddish center with black ring.

Fig. 4, (row 2, left). Eosinophil. Red granulation throughout cytoplasm encircling yellowish preformed granules of the cell.

Fig. 5, (row 2, middle). Lymphocyte, basophilic. Intermediate to large in size. Dark blue to light blue cytoplasmic granules with several azurophilic granules. Heterophil below.

Fig. 6, (row 2, right). Thrombocytes. Vacuolated cytoplasm. Reddish granules in a small mass in one thrombocyte.

Fig. 7, (row 3, left). Lymphocyte, small. Azurophilic granules relatively large.

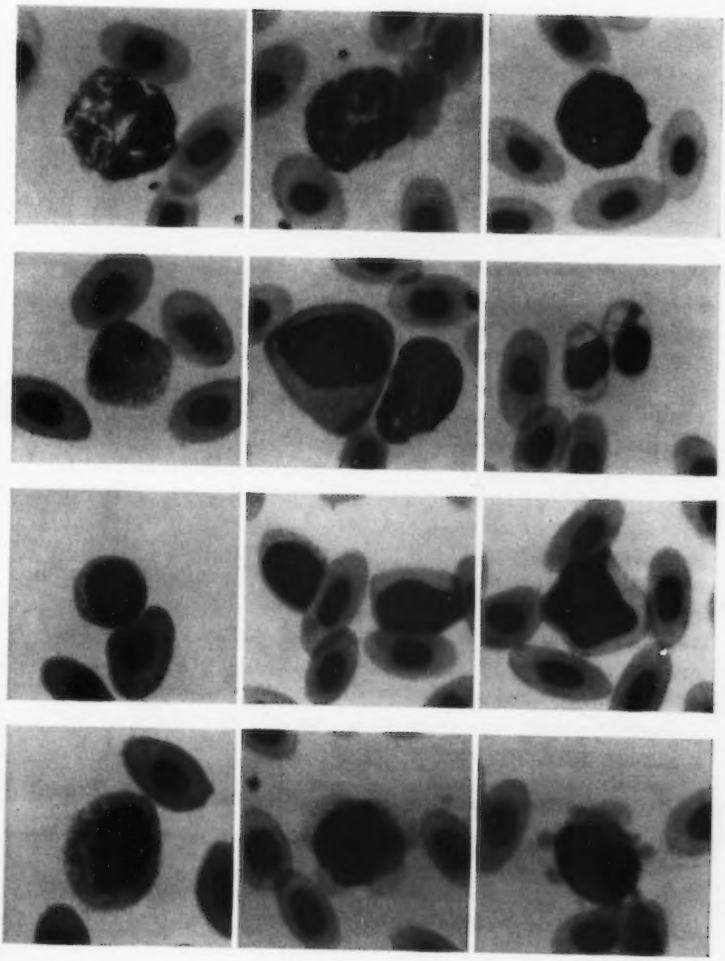
Fig. 8, (row 3, middle). Lymphocytes, small. Several small vacuoles present in one cell. Azurophilic granules present.

Fig. 9, (row 3, right). Lymphocyte, mature. Intermediate in size. Pinpoint azurophilic granules present.

Fig. 10, (bottom row, left). Monocyte A. Granular cytoplasm relatively wide. One small projection of ectoplasm present, beginning of monocyte B. Compare position of nucleus, cytoplasm and azurophilic granules with monocytes B in middle and right.

Fig. 11, (bottom row, middle). Monocyte B. Ectoplasm with several indentations. Endoplasm and azurophilic granules condensed around nucleus.

Fig. 12, (bottom row, right). Monocyte C. With ectoplasmic tags. Endoplasm and azurophilic granules condensed around nucleus. Monocyte B without ectoplasm is classified as monocyte C.



Heterophils (figure 2). The preformed granules may be sharp-pointed to blunt-ended rods staining red-brown to dark-brown. A few heterophils may contain small, black granules seemingly superimposed on the preformed granules and on the nucleus. With critical focusing most of these black granules will be seen to conform in size, shape and position to the inner granule[2] present in the preformed granules. Some of these black granules appear to conform in size and shape to small preformed granules. A round, black granule may be observed in some scattered granules in the vicinity of a crushed heterophil, demonstrating that they are not artifacts. The color of the cytoplasm of the heterophils varies from apricot to light-gray.

Basophils (figure 3). These cells are generally filled with round and oval, small and large preformed granules, so closely packed as to make the cytoplasm appear homogeneous and uniformly reddish-purple, dotted with black. Most of these granules show some black color, pinpoint to the full size of the granule. By critical focusing, narrow, black rings with light-red centers can be observed. Some of these rings appear to be formed by the joining of the points of black color present in the adjacent granules. Other rings are formed by the solid-black preformed granules. An occasional basophil will show an area in the cytoplasm devoid of preformed granules but with its gray-blue granules. A colorless area, the size of a basophil granule, may show only one or two pinpoint reddish granules. A basophil may be seen with one or two irregularly shaped, clear areas probably the result of contraction of the cytoplasm. Most basophils show one or more black preformed granules superimposed on the nucleus.

Eosinophils (figure 4). The round, preformed granules stain yellow to orange in color, and in most eosinophils they may be observed to be encircled or partially encircled with minute, discrete, round or angular red granules giving the cells a compact, mosaic pattern of light centers with red rings. An occasional eosinophil is present without these red granules. In this latter cell the cytoplasm is light-blue in color. Eosinophils may be seen with these red granules so numerous as

to practically obscure the outlines of the preformed granules giving the cell the appearance of having a yellow cytoplasm peppered with red. The presence of these red granules in the cytoplasm aids in differentiating the eosinophil from the heterophil.

Thrombocytes (figure 6). The gray-blue granular cytoplasm of the elliptical or round, mature thrombocyte nearly always contains one or more vacuoles. The cytoplasm surrounding these vacuoles may be narrow or wide. Pinpoint, round, light-red granules fill the cytoplasm. A single row of these granules may be present in the thin line of cytoplasm encircling some vacuoles. Aggregations of the red granules may be located between or within some of these vacuoles. The oval or round nucleus may be centered or eccentric in position in the cell. An occasional thrombocyte, without vacuoles, may contain deep-blue, instead of the usual gray-blue, cytoplasmic granules. A few or no red granules may be present in this cell.

Lymphocytes (figures 7, 8, 9). The color of the cytoplasm in the small lymphocytes varies from cell to cell in the same preparation and from bird to bird. The cytoplasmic granules in these small, mature cells may stain light-blue to gray-blue. Young lymphocytes, intermediate and large, occasionally seen in the blood of young chickens, have cytoplasms with deep-blue granules, whereas, the cytoplasm in mature lymphocytes of corresponding size, stain gray-blue to light-gray. The outer periphery of the cytoplasm of some small lymphocytes shows a narrow zone of dark-blue color fading to light-blue near the nucleus. In some small lymphocytes one or more vacuoles may be present, and frequently a small, round, red granule may be seen within a vacuole. Azurophilic granules are nearly always found in the cytoplasm of small lymphocytes, also in the intermediate and large cells, young or mature. These azurophilic granules differ from those present in the monocytes in being fewer in number, more dispersed in position, more round in shape, more uniform in size, and lighter in color. An occasional small lymphocyte may show several, short cytoplasmic tags projecting from the cell.

Monocytes (figure 10). The chromatin of the nucleus stains black-purple to almost black and the nucleoplasm reddish-purple. One of the distinguishing characteristics separating mature monocytes from mature lymphocytes is the conspicuous black color in the chromatin of the nucleus of the monocyte, in contrast to the purple-red color in the chromatin of the nucleus of the lymphocytes. Also the chromatin pattern is more conspicuous in the monocyte than in the lymphocyte. The monocytes in the peripheral blood of the chicken vary considerably in their morphology, particularly in respect to the character of the cytoplasm. The cytoplasm in some monocytes shows an endoplasm and an ectoplasm. The ectoplasm appears hyaline, but with careful focusing it can be seen to be composed of minute, blue-gray granules. This ectoplasm may be relatively narrow or wide, with a smooth, shallow or deeply indented outline or serrated with a fringe of ectoplasmic tags. Some monocytes may be completely denuded of ectoplasm, the visible cytoplasm being the former endoplasm. Azurophilic granules are a constant and typical feature of the monocytes. Rarely will these granules be seen in the ectoplasm. The monocytes in the peripheral blood of normal young chickens may be conveniently classified, for descriptive purposes, in 3 groups, Monocytes A, B and C, according to the appearance and position of the nucleus, the cytoplasm and azurophilic granules.

Monocyte A. (figure 10). Small, irregularly shaped azurophilic granules are distributed quite uniformly throughout the wide, gray-blue, granular cytoplasm. Ectoplasm is not visible. The round or oval nucleus may be centered or slightly eccentric in position.

Monocyte B. (figure 11). This is the most common monocyte present in the blood film of a young chicken. The first visible indication of ectoplasm in the monocyte in this staining procedure, is the presence of a knob of hyaline, gray-blue cytoplasm projecting from the periphery of the cell. The wide band of ectoplasm frequently seen in monocytes appears to develop by the coalescence and expansion of cytoplasmic projections of varying size and number. The round to oval

nucleus and the still wide, granular endoplasm remain relatively centered in the cell. Other monocytes show the nucleus to be more compact, indented and eccentrically located near or against the cell membrane with its endoplasm narrowed and condensed against the nucleus, completely or partially encircling it. The endoplasm becomes darker-blue in color, and the azurophilic granules more aggregated as the ectoplasm appears to "breakup" into numerous, small projections or tags assuming a serrated fringe of blebs, racquets, and thin threads or thin threads alone.

Monocyte C (figure 12). This cell is monocyte B denuded of its visible ectoplasm. Monocyte C, without ectoplasm, is easily distinguished from monocyte A, without ectoplasm, by differences in the appearance, position and color of its cytoplasm, its aggregation of azurophilic granules, and the shape, size, compactness and eccentric position of its nucleus.

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SUMMARY

1. A procedure using May-Greenwald, Wright and Phloxine B in combination for staining peripheral blood cells of the chicken is described. The preparation of the stain, buffer solutions and the steps in the procedure for staining the fixed blood films are detailed.

2. A procedure for making films of chicken blood on waxed slides with glass blocks is described. Advantages and comments are given on the steps in the procedure for making the blood films.

3. Differential staining characteristics of the erythrocytes, heterophils, basophils, eosinophils, thrombocytes, lymphocytes and monocytes observed in stained fixing preparations of peripheral blood cells of apparently normal 5 and 10-week-old chickens are described.

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SALMONELLA ORGANISMS IN ANIMAL PRODUCTS USED IN POULTRY FEEDS*

By JOHN R. WATKINS, A. I. FLOWERS, and L. C. GRUMBLES

Department of Veterinary Microbiology
Texas Agricultural Experiment Station
College Station, Texas

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MEMBERS of the genus *Salmonella* were first recognized as a cause of disease in poultry in the late 19th century. Since then the rapid expansion of the poultry industry has magnified the importance of this genus. All ages of turkeys and chickens are attacked by one or more of its mem-

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bers. Losses from infections by *Salmonella pullorum* and *Salmonella gallinarum* have been reduced but not eradicated by various tests and control measures. Control of other members of the genus in poultry have been less satisfactory because of their wide distribution, lack of host specificity and variable antigenic structure.

On occasion, infections appear without apparent source. Because *Salmonella* species are associated with animals, that portion of the feed of animal origin was suspected. In 1955, Erwin[1] reported the isolation of *Salmonella oranienburg* from 3 of 203 samples of prepared poultry feeds. In 1956 the poultry disease diagnostic laboratory of the Texas Agricultural Experiment Station at College Station isolated *Salmonella panama* from one sample and *Salmonella newport* from another sample of poultry by-products.

Numerous other investigators have isolated *Salmonella* from products of animal origin. In many instances these products are used in prepared poultry feeds. Felsenfeld *et al.*[2] isolated *Salmonella* from market beef, dried eggs, poultry, pork and hamburger. The highest incidence of contamination was in pork and hamburger. Walker,[7] making a survey of the occurrence of *Salmonella* in organic fertilizers found 40 percent of the samples contaminated. Galton *et al.*[3] examined 159 samples of 22 brands of dehydrated dog foods and found only meals contaminated. Muller[5] in Denmark cultured 1,093 samples of imported meat and blood meal and bone meal between 1949 and 1954. Salmonella organisms were isolated from 4.5 percent of the samples.

When it appeared that animal by-products might be the source of *Salmonella* infections in poultry, it became important to determine the frequency of occurrence of the organisms in such material. This was the objective of this study. The results revealed that numerous species of *Salmonella* were frequently present in animal by-products intended for use in feeds.†

† After this work was completed, a paper bearing on the same subject appeared by C. I. Boyer, Jr., D. W. Bruner, and J. A. Brown, *Salmonella* organisms isolated from poultry feed, Avian Diseases, *2*, 346, 1958. Our work is an essential agreement with the findings of these authors.

MATERIALS AND METHODS

Two hundred samples of processed animal by-products for use in feeds were obtained from several sources in the state through cooperation with the Texas Feed Control Service. The Feed Control Service set aside a portion of the desired samples as they were presented for composition analysis.

The following media were used in attempts to isolate *Salmonella*: tetrathionate and selenite broths; brilliant green, SS, MacConkey's and bismuth sulfite agars. MacConkey's agar was used on five of the samples. Selenite broth and bismuth sulfite agar were used on 30 of the samples.

Two grams of each sample were inoculated into each of five tubes containing 15 ml of tetrathionate broth and five tubes containing 15 ml of selenite broth and were incubated for 24 hours at 37.5 C. One-half plate of each of the solid media was streaked with one standard bacteriological loop of material from each tube of enrichment media. After 24 hours of incubation, one or two colonies grossly resembling *Salmonella* were selected from each plate and inoculated into lactose carbohydrate broth. If lactose fermentation did not occur after 24 hours incubation, a transfer was made to five carbohydrates (dextrose, maltose, lactose, sucrose, and mannitol), SIM medium and urea agar. After 7 days incubation, if the carbohydrate fermentation, urea, and SIM reactions were characteristic for one of the *Salmonella* group, an inoculum was taken from lactose broth and transferred to a tryptose agar slant. This culture was sent to the Communicable Disease Center, Chamblee, Georgia for serological typing.

The samples found to be contaminated by *Salmonella* were subjected to heat to determine the heat tolerance of the organisms. Five grams of the material were suspended in 25 ml of tetrathionate broth, exposed to 65 C for 35 minutes, and subjected to the same cultural procedures as previously outlined. The temperature and time used were slightly above those given by Merchant and Packer[4] as sufficient to kill *Salmonella*. The Texas State Department of Health[6] recommends that processing be done at 212 F and 15 pounds of pressure for one hour.

To determine whether contamination may have resulted from faulty cooking or improper handling procedures, a personal visit was made to seven processing plants. Three plants (A, D, E) from whose product *Salmonella* had been isolated and four from whose product *Salmonella* had not been isolated, were chosen for observation. Additional samples were obtained and each of the seven plants was observed for possible correlation between the presence of *Salmonella* in the finished product and the methods used to prepare the product. When possible, the samples were taken from the top layer in the percolation vats after the cooked material had cooled. This was not possible at Plants A, D, and G. Material was collected from a bulk storage pile at Plant A, from the bottom of a recently emptied vat at Plant D, and from a solvent extraction cooker at Plant G. Portions were taken from several places in the bulk material and placed in a sterile container. Isolation attempts were made in a manner similar to that previously described.

RESULTS

Salmonella species were isolated from 37 of 200 samples of poultry and animal by-products meals (18.5 percent). Thirty-one isolations were made from animal by-products other than poultry. Five isolations were made from thirty samples of poultry by-product meal and one was made from 18 samples of hydrolyzed feather meal.

The species of *Salmonella* isolated and the media used in culturing each sample from which an isolation was made are given in table 1. All isolations were typed serologically and 28 species were identified (table 4). A majority of the samples were contaminated with several species. Eight species were isolated from sample 157.

Six *Samonella* species were isolated from five of the 30 initial samples using tetrathionate broth. Only one isolation was made from the same samples using selenite as the enrichment broth. MacConkey's agar was not used for more than five samples because it did not restrict lactose fermenting bacteria, making it impossible to differentiate colony types.

TABLE I

Sample number, species of *Salmonella* isolated, and media on which isolated

Sample number	Type of material	Species isolated	Solid S S	Media B. G.*	(1) B. S.*	Tetra (2)
102	Poultry by-products	*S. cubana*	1″	not used	not used	1#
106	Poultry by-products	*S. cubana*	2		2″	2
		S. worthington	1			1
118	Poultry by-products	*S. newington*	1	1″	1	1
		S. binza				
	Feathers		1	1		1
129	Poultry by-products	*S. illinois*	2	2		2
	Feathers					
130	Poultry by-products	*S. newington*	1			1
		S. senftenberg		1	2	2
	Feathers	*S. illinois*	1	1		1
134	Meat and bone scraps poultry by-products	*S. worthington*	1	1	—	1
142	Digester Tankage	*S. lexington*	1	1		1
144	Meat and bone scraps	*S. montevideo*	2	2		2
153	Meat and bone scraps	*S. senftenberg*	1	1		1
157	Meat and bone scraps	*S. senftenberg***	1	1		1
		*S. schwarzen-grund***	1			1
		*S. infantis***	1			1
		*S. derby***		1**		1
		*S. cerro***		1**		1
		*S. montevideo***				
		*S. lexington***				
		*S. minnesota***				

* B. G. — Brilliant Green, B. S. — Bismuth Sulfite, Tetra. — Tetrathionate

(1) MacConkey's agar was used for samples 103-108. An isolation was made from sample 106.

(2) Selenite broth was used as an enrichment for samples 103-130. Isolations on followup media were successful from sample 106.

″ Number of plates of five per sample from which isolations were made.

Total number of isolations of each specie from a sample following enrichment in tetrathionate.

** These species were isolated from the two plates of brilliant green indicated.

TABLE I (Continued)

Sample number, species of *Salmonella* isolated, and media on which isolated

Sample number	Type of material	Species isolated	Solid S S	Media B. G.	(1) B. S.	Tetra (2)
158	Meat and bone scraps	*S. anatum*	1	1		1
180	Meat and bone scraps	*S. give*	1	1		1
		S. cubana		1		1
		S. montevideo	2	2		2
		S. cerro	1			1
182	Meat and bone scraps	*S. minnesota*		1		1
		S. montevideo	2			2
		S. worthington	1	3		3
		S. illinois		1		1
		S. oranienberg	1			1
183	Meat and bone scraps Blood meal	*S. anatum*	1	1		1
197	Meat and bone scraps	*S. infantis*	1	1		1
		S. anatum	1	1		1
		S. seigburg	1	1		1
202	Meat and bone scraps Poultry by-products	*S. tennessee*	1	1		1
205	Meat and bone scraps	*S. cubana*	1	1		1
206	Meat and bone scraps	*S. senftenberg*	1	1		1
207	Meat and bone scraps	*S. seigburg*	1	1		1
224	Meat and bone scraps	*S. infantis*	2	2		2
		S. seigburg	3	3		3
228	Hydrolyzed Feathers	*S. infantis*		1		1
239	Digester Tankage and bone	*S. gaminara*	1			1
246	Meat and bone scraps	*S. montevideo*	2	2		2
262	Meat and bone scraps	*S. cerro*		1		1
		S. bareilly	1			1

TABLE I (Continued)

Sample number, species of *Salmonella* isolated, and media on which isolated

Sample number	Type of material	Species isolated	Solid SS	Media B. G.	(1) B S.	Tetra (2)
270	Meat and bone scraps	*S. illinois*		1		1
		S. cerro	1	1		1
		S. senftenberg	2	2		2
		28:Y	1	1		1
278	Meat and bone scraps	*S. worthington*		2		2
		S. bredeney	1	1		1
		S. cerro	1	1		1
		S. san diego	1			1
		S. pomona		1		1
279	Meat and bone scraps	*S. anatum*		1		1
		S. senftenberg	1	1		1
280	Meat and bone scraps	*S. anatum*		2		2
		S. minnesota	1			1
281	Meat and bone scraps	*S. cubana*		1		1
		S. montevideo	3	2		3
		S. worthington	1			1
		S. lexington		1		1
285	Meat and bone scraps	*S. canoga*	1	1		1
		S. illinois	1			1
		S. lexington	1	1		1
291	Meat and bone scraps	*S. anatum*	2	1		2
		S. infantis	1	2		2
292	Digester Tankage	*S. infantis*	1	1		1
		S. illinois	2	2		2
		S. oranienburg		1		1
293	Meat and bone scraps	*S. illinois*		1		1
		S. oranienburg	1			1
		S. senftenberg		1		1
300	Meat and bone scraps	*S. anatum*		1		1
		S. montevideo	1	1		1
		S. minnesota	1			1
302	Meat and bone scraps	*S. california*	2	3		3
		S. lexington	1	1		1
		S. senftenberg	2			2
		S. anatum		1		1
303	Meat and bone scraps	28:Y	2	2		2
		S. senftenberg	1	1		1
		S. infantis	2	2		2
		S. illinois	1	1		1
305	Meat and bone scraps	*S. illinois*		1		1
		S. infantis	1			1
		S. senftenberg	1	1		1

Salmonella organisms were isolated from three of 30 samples with bismuth sulfite agar but were isolated from four of these samples on each of the other media used. Difficulty in differentiating *Salmonella* and *Proteus* colonies necessitated additional attempts at identification.

Brilliant green and SS agar appeared to have approximately equal value. On one occasion (sample 228) an isolation was made on brilliant green but not on SS agar. In another instance, (sample 229) the reverse was true. This only occurred when the concentration of the organisms was low. SS agar was more inhibitory to other contaminants than

TABLE II

Results of culturing selected samples of products taken at seven processing plants

Plant	Number of samples	Type of material	Source	Results of Culture
A	2	Feather meal	Bulk storage on floor	Negative
	6	Poultry meat and bone scraps	Bulk storage on floor	Negative
B	4	Meat and bone scraps	Percolation vat	Negative
C	3	Meat and bone scraps	Percolation vat	Negative
	2	Blood meal	Percolation vat	Negative
D	4	Meat and bone scraps	Scrapings from bottom of percolation vat	*Salmonella madelia* *Escherichia* *Proteus*
E	5	Meat and bone scraps	Percolation vat	*Escherichia* *Alkaligenes* *Proteus*
F	5	Meat and bone scraps	Percolation vat	*Escherichia* *Alkaligenes* *Proteus*
G	5	Meat and bone scraps	Solvent extraction cooler	*Escherichia*

brilliant green agar, but had a tendency to allow lactose fermenters to grow without visible color change. This made colony differentiation difficult on occasion. Colonies were more easily selected from brilliant green because of a greater color contrast, but there was a marked failure of inhibition of many *Proteus* and *Alkaligenes* species. These genera produced pigment similar to that of the *Salmonella*. In the early phase of this investigation these were thought to be *Salmonella* colonies and further cultural work was required to prove differently. Later it was noticed that colonies of these genera had a dry crusty appearance after 48 hours incubation rather than the smooth, homogenous appearance of the *Salmonella*.

This study indicates that re-contamination following cooking is primarily responsible for the presence of *Salmonella* organisms in animal by-products. *Salmonella* could not be reisolated from known contaminated samples following heat treatment. No isolations were made from samples taken at

TABLE III

Species of paratyphoid isolated from chickens and turkeys in Texas July, 1956 to December 30, 1958

Species:	Number Isolations Turkeys	Chickens
S. javiana	8	0
S. newport	8	2
S. typhimurium	7	0
S. san diego	7	0
S. give	7	1
S. st. paul	5	0
S. muenchen	4	0
S. panama	4	0
S. oranienburg	3	0
S. heidelburg	3	0
S. anatis	2	0
S. derby	1	0
S. montevideo	1	1
S. infantis	1	0
S. berta	1	0
S. hamiltonia m.sp.	1	0
S. blackley	1	0
Group E	1	0
S. thompson	0	1
TOTAL	65	5

the plants unless the material had been handled following its discharge from the cooker into the percolation vat. One isolation was made from material scraped from the bottom of a percolation vat. The results of culturing the samples taken at the plants are given in table 2.

There appeared to be a relationship between the method of handling and the absence or presence of recontamination in two plants. At Plant A, raw wastes leaked onto the floor and contaminated the cooked material as it was discharged. Plant G used a solvent cooker to extract the residual fat. The remaining material was then discharged directly into an auger conveyor system for transportation to a separate sacking building, thus elminating recontamination from handling equipment or contact with raw materials.

TABLE IV

Salmonella isolated from animal by-product used in poultry feeds

Species	Number of times recovered	Species	Number of times recovered
S. senftenberg	11	*S. binza*	1
S. illinois	8	*S. muenchen*	1
S. montevideo	8	*S. schwarzengrund*	1
S. anatum	8	*S. derby*	1
S. infantis	7	*S. give*	1
S. cerro	5	*S. tennessee*	1
S. lexington	5	*S. gaminara*	1
S. worthington	5	*S. bareilly*	1
S. cubana	4	*S. bredeney*	1
S. minnesota	4	*S. san diego*	1
S. newington	2	*S. canoga*	1
S. oranienburg	2	*S. california*	1
S. seigburg	2	*S. panama*	1
28:Y	2	*S. pomona*	1

DISCUSSION

Animal by-products may serve as a source of *Salmonella* infection for poultry if used in feeds. A comparison of table 3 with table 4 shows that one-half of the species isolated from chickens and turkeys in the state were present in animal by-products. In all probability, if a greater number of samples had been assayed or if additional colonies had been selected for identification from the samples investigated, there would

have been a closer correlation between the species isolated from feed ingredients and those recovered from chickens and turkeys. One of the species listed, *Salmonella senftenberg*, was diphasic. Edwards reported in a personal communication that it had not been typed previously at C.D.C. from a source in the United States, and only once from a foreign source.

The use of two agar media increased the number of isolations. Probably this was not an indication of the superiority of one medium over the other, but merely an increased probability of success due to a greater number of attempts from a given sample. This bears out the findings of Galton *et al.*[3] that a given amount of material in numerous tubes of enrichment media resulted in a greater number of isolations than the same amount of material in a single large tube of enrichment media. The greater number of isolations of a particular species on one of the media can not be interepreted as a preference for one type of medium because only one colony was picked from each half of a plate. More colonies were not picked because of the pyramiding work-load. Either brillliant green or SS agar would serve well in a routine examination of these products. Tetrathionate broth appeared to be definitely superior to selenite broth because selenite inhibited growth excessively.

Urea agar was an excellent medium for detecting *Proteus* rapidly. In one instance, however, seven days were required for a culture to demonstrate urease production.

The inspection of the processing plants made during the visit to them could not uncover all possible sources of recontamination. However, gross violations of routine sanitary procedures were obvious. The small plants were more often guilty of this because the workers were used in various areas to fully utilize their time. Facilities for disinfecting or changing boots were not evident. Even though men from contaminated areas did not necessarily enter the percolator vats, their presence on the walkways used by men who did, could serve as a source of recontamination. Once the organisms were introduced into the percolation vat, the warm meat scraps

could undoubtedly serve as a culture medium. The organism would then be present in the final product. The presence of *Salmonella madelis* in the scrapings taken from the bottom of the percolation vat at Plant D could indicate reintroduction or a survival of the organism under a protective crust which might occur if freshly cooked meat scraps were discharged into a partially emptied vat.

SUMMARY

Two hundred samples of animal by-products were cultured for *Salmonella* organisms. Thirty seven samples (18.5 percent) were found to be contaminated. A majority of the samples were contaminated by more than one species. Twenty-eight different species of *Salmonella* were isolated.

Heat studies conducted on the contaminated samples demonstrated the susceptibility of the 28 species to a temperature of 65 C for 35 minutes. *Salmonella* organisms were not found in freshly cooked material taken during a personal visit to seven processing plants. *Salmonella madelia* was isolated from the scrapings taken from the bottom of a percolation vat. It would appear that *Salmonella* organisms are introduced into the by-products as a result of recontamination of the cooked product in the processing plant.

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AVIAN INFECTIOUS HEPATITIS
III. TREATMENT OF CHICKENS IN EGG PRODUCTION*†

By MARTIN SEVOIAN and B. W. CALNEK
Department of Veterinary Science
University of Massachusetts

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THE effects of various drugs on the infectious hepatitis agent (IHA) have been reported.[5] These drugs have been evaluated in chicken embryos and in young chickens. Subsequent reports have established the causal agent to be a vibrio.[1,2,3]

METHODS AND MATERIALS

In two separate trials, the relative efficacies of furazolidone and dihydrostreptomycin sulfate for the control of infectious hepatitis in laying chickens were investigated.

In trial I, two hundred White Leghorn yearling chickens, apparently healthy, were divided and treated in the following manner.

Birds	Infected	Medication
50	+	None
50	+	Furazolidone
50	+	Dihydrostreptomycin sulfate
16	—	Furazolidone
17	—	Dihydrostreptomycin sulfate
17	—	None

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Each of the infected groups was kept in a separate house for 5 weeks prior to infection and 8 weeks thereafter. Extreme care was taken to keep the non-inoculated groups free of contamination. Hepatitis isolates, #79, 1297, 9683 and 9805 harvested from yolk sacs of infected embryos were used as a pool to inoculate each bird intra-abdominally with 10,000 ELD_{50}. These isolates, as well as all other IHA isolates maintained at this station, have been grown on blood agar and appear to be culturally similar if not identical to those previously described.[1,2,3] Drugs were administered at 48 hours postinoculation. Dihydrostreptomycin sulfate (250 mgm per bird) was injected intramuscularly and furazolidone (300 gms per ton of feed) was fed for 6 consecutive days. The birds were trapnested and individual production records were kept. Birds were selected at random twice weekly and sacrificed for examination and culture from the 6th to the 58th day after inoculation. Suspensions of liver, isolation attempts, titrations and serial passages were made as previously described.[4,5]

In trial 2 there were 171 Rhode Island Red 7-month-old chickens divided into groups as in trial 1. Drugs were administered at 7 days postinoculation when the disease was known to be well established. Dihydrostreptomycin (250 mg per bird) was injected as in trial 1 but the level of furazolidone was lowered to 250 gms per ton of feed and fed for 5 consecutive days. Birds were selected at random weekly and sacrificed for examination and culture from the 4th to the 95th day after inoculation. Suspensions of liver, isolation attempts, titrations and serial passages were made as previously described.[4,5] Previous work at this station had indicated that bile contained greater concentrations of the IHA than other sites. Therefore bile was aseptically withdrawn with a syringe and needle and placed directly on blood plates and into broth suspension for chicken embryo inoculations. Blood plates were incubated under reduced oxygen tension.

Eggs from the infected controls were incubated and examined for fertility and embryo deaths to 20 days. Yolk was harvested from embryos that died on both first and second passage and was repassed.

RESULTS

About 20 percent of the infected controls had signs of morbidity whereas mortality was less than 5 percent in either trials 1 or 2. Isolations of the IHA were made as early as 4 days postinoculation and well-developed gross lesions were present at 7 days postinoculation.

Isolations in chicken embryos. Table 1 indicates the mortality of chicken embryos inoculated with suspensions of livers from experimentally infected laying chickens (trial 1).

TABLE I

Trial 1. Chronological mortality of chicken embryos inoculated with 25 positive cultures from hens experimentally infected with avian infectious hepatitis

Days post inoc.	No. dead on 1st passage*	No. dead on 2nd passage
3	14	60
4	29	63
5	41	18
6	15	3
7	1	1
8	2	1
9	1	0
10	0	0
11	0	0
12	1	0
13	0	0
14	1	0
Total dead/Total inoc.	105/144	146/147

* 6 eggs inoculated with each of 25 cultures. During first 2 days 6 embryos dead on 1st passage and 3 embryos dead on 2nd passage due to non-specific causes.

Positive samples usually caused embryo deaths within 6 days postinoculation on the first passage. In nearly all instances, those cultures not causing deaths in all embryos on the first passage did so on the second (table 2). Sporadic mortalities in the first passage apparently were the result of low concentrations of the agent in some liver cultures. As seen in table 3, the inoculation of embryos with negative samples also resulted in sporadic non-specific mortalities in some instances. In these cases, embryo deaths on second passage were non-specific and sporadic as on first passage or non-existent.

Table 5 contains the results of chronological isolation attempts in chicken embryos of suspensions of livers from experimentally infected hens treated with dihydrostreptomycin sulfate and furazolidone. No isolations were made from the treated birds, whereas nearly 50 percent of the infected controls yielded the IHA on culture. One isolation was made from the non-inoculated, non-medicated group.

TABLE II

Trial 1. Mortalities of chicken embryos inoculated with 25 positive samples from hens experimentally infected with avian infectious hepatitis

	Dead/inoculated					
	1/6	2/6	3/6	4/6	5/6	6/6
1st embryo passage	3*	4	1	2	1	14
2nd embryo passage	0	0	0	0	1	24

* Number of samples (birds) where 1 of 6 inoculated embryos died, etc.

In trial 2, data of isolations with suspensions of liver or bile or both are summarized in table 6. From the time when treatment was instituted 7 days postinoculation, recovery of the IHA was made from 100 percent of the infected controls as compared with 15 percent of the group treated with furazolidone. No isolations were made from the dihydrostreptomycin-treated group and the medicated and non-medicated, non-infected groups. Suspensions of liver on blood agar did not yield growth whereas parallel cultures of undiluted bile resulted in growth from 55 percent of the samples as compared to 100 percent isolations in chicken embryos. However, when bile was diluted 10^{-1} or greater in nutrient broth, no growth resulted on blood agar.

Bile from 14 infected birds when titrated in chicken embryos had IHA titers of 0.2 ml x $10^{-2.5}$ to $10^{-8.4}$ ELD_{50}. Titrations of liver suspensions from the same birds resulted in titers of 0.2 ml x $10^{-1.5}$ to $10^{-6.1}$ ELD_{50} (table 4). Where birds were established to be positive by isolations in chicken embryos from suspensions of liver or bile or both, undiluted bile yielded the IHA 100 percent of the time as compared to only 40 percent from liver samples. When yolks from chicken embryos dead

TABLE III

Chicken embryo mortalities on primary egg passage from the avian infectious hepatitis agent of cultures from experimentally infected hens treated with dihydrostreptomycin and furazolidone. Trial 1

	Isolations													
	Dihydrostrept.*		Furazol.†		Infected controls		Noninfected controls						Total	
							Dihydrostrept.		Furazol.		Non-medicated			
#dead/#inoc.	Pos.	Neg.	Pos	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
0/6		31‡		43		19		14		15		14		136
1/6		11		5	3	6		3		2		1	3	28
2/6		1			3			1			1		4	2
3/6					1								1	
4/6					2								2	
5/6					1								1	
6/6					14								14	
Total		43		48	24	25		18		17	1	15	25	166

* — 250 mgm of dihydrostreptomycin per bird 48 hours postinoculation
† — 300 gms of furazolidone per ton of feed for 6 days beginning 48 hours postinoculation
‡ — Number of samples (birds)

from the IHA were cultured on blood agar, growth resulted from approximately 33 percent of the samples.

Chickens which were severely affected (20 percent) by the disease had stopped laying whereas less affected birds (80 percent) continued to lay but in an interrupted fashion. Some ova were in the process of retrogression and resorption.

More than 1200 eggs from known infected mated chickens were collected over a period of 8 weeks postinoculation. Upon incubation, only 66 percent were found to be fertile. Yolk cultures of all embryos dead (13 percent of fertile eggs) up to 20 days of incubation were placed in parallel on blood agar and into the yolk sacs of chicken embryos for passage. Cultures of eggs dead on second passage were repassed. No isolations were made.

TABLE IV

Titrations of the infectious hepatitis agent in chicken embryos from cultures of liver and bile. Trial 2

Bird	Weeks postinoc.	ELD_{50}*	
		Liver	Bile
A	3	4.3	6.5
B	4	5.8	7.6
C	5	3.5	3.5
D	6	4.6	7.2
E	7	1.5	4.9
F	8	6.1	3.6
G	9	5.5	8.2
H	10	4.2	7.4
I	11	4.0	8.4
J	12	2.3	7.6
K	13	2.0	2.8
L	14	1.5	2.5
M	15	1.5	2.5
N	16	3.6	2.5

* Titers represented as reciprocal of the log.

Cultures of surviving embryos from titration studies were given 3 blind passages in chicken embryos in order to study whether or not sublethal embryonic infections occurred with the IHA. In no instance was the IHA detected in surviving embryos.

DISCUSSION

Early reports on the effectiveness of furazolidone and

TABLE V

Results of chronological reisolation attempts in chicken embryos of the infectious hepatitis agent from experimentally infected hens treated with dihydrostreptomycin and furazolidone. Trial 1

Days post inoc. necropsy	Positive samples (birds)/Total samples*					
				Noninfected controls with		
	Dihydrostrept.†	Furazol.‡	Infected controls	Dihydro.	Furazol.	Non medicated
6	0/2	0/3	0/3	0/1	0/1	0/1
9	0/2	0/3	1/3	0/1	0/1	0/1
13	0/2	0/2	1/3	0/1	0/1	0/1
16	0/3	0/3	2/3	0/1	0/1	0/1
20	0/3	0/3	1/3	0/1	0/1	0/1
23	0/3	0/3	1/3	0/1	0/1	0/1
27	0/2	0/3	1/3	0/1	0/1	1/1
30	0/3	0/3	2/3	0/1	0/1	0/1
34	0/3	0/3	2/3	0/1	0/1	0/1
37	0/3	0/3	2/3	0/1	0/1	0/1
41	0/3	0/3	1/3	0/1	0/1	0/1
44	0/2	0/3	2/3	0/1	0/1	0/1
48	0/3	0/3	2/3	0/1	0/1	0/1
51	0/3	0/3	2/3	0/1	0/1	0/1
55	0/3	0/3	2/3	0/1	0/1	0/0
58	0/3	0/4	2/4	0/1	0/3	0/2
Totals	0/43	0/48	24/49	0/16	0/18	1/17

* — contaminated samples not counted
† — 250 mgm of dihydrostreptomycin per bird at 48 hours postinoculation
‡ — 300 gms of furazolidone per ton of feed for 6 days beginning 48 hours postinoculation

TABLE VI

Results of chronological reisolation attempts in chicken embryos of the infectious hepatitis agent from experimentally infected hens treated with dihydrostreptomycin and furazolidone. Trial 2

Days post inoc. necropsy	Positive samples (birds)/Total samples*					
				Noninfected controls with		
	Dihydrostrept. †	Furazol. ‡	Infected controls	Dihydro.	Furazol.	Non medicated
4	4/4	2/3	1/3	0/1	0/1	0/1
Treated 7th day						
11	0/3	0/3	3/3	0/1	0/1	0/1
18	0/3	0/3	3/3	0/1	0/1	0/1
25	0/3	2/4	3/3	0/1	0/1	0/1
32	0/3	1/3	3/3	0/1	0/1	0/1
39	0/3	1/3	3/3	0/1	0/1	0/1
46	0/3	1/3	4/4	0/1	0/1	0/1
53	0/3	0/3	3/3	0/1	0/1	0/1
60	0/3	0/3	3/3	0/1	0/1	0/1
67	0/3	0/3	3/3	0/1	0/1	0/1
74	0/3	1/3	3/3	0/1	0/1	0/1
81	0/3	0/3	3/3	0/1	0/1	0/1
88	0/3	0/3	3/3	0/1	0/1	0/1
95	0/3	0/3	3/3	0/1	0/1	0/1
Totals	0/39	6/40	40/40	0/13	0/13	0/13

* — contaminated samples not counted
† — 250 mgm of dihydrostreptomycin per bird 7 days postinoculation
‡ — 250 gms of furazolidone per ton of feed for 5 days beginining 7 days postinoculation

dihydrostreptomycin sulfate against the IHA in chicks have resulted in extensive use of these drugs in field outbreaks involving laying chickens. Experimental results herein reported lend support to these recommendations. In trial 1, both drugs apparently gave excellent protection although the isolation rate from the infected controls was only 48 percent. Though apparently healthy birds were selected for the experiment, an isolation was made from one bird in the non-inoculated, non-medicated group. From our experience with infectious hepatitis in flocks in Massachusetts, the disease is widespread but its significance varies much from flock to flock. To recognize infectious hepatitis in an occasional bird in a given flock is not unusual. Therefore it was not surprising, though unexpected, to make an isolation from this group of birds. In trial 2, dihydrostreptomycin sulfate gave somewhat better control than did furazolidone (250 gm per ton). The isolation rate from the infected controls from the time of medications was 100 percent. Neither relapse nor reinfection resulted over a period of 3 months in the infected group treated with dihydrostreptomycin sulfate whereas a relapse appears to have taken place in part of the group treated with furazolidone.

Chicken embryos were a better medium than blood agar for the propagation of the IHA. Apparently larger concentrations of this agent were needed for growth on blood agar. It is conceivable, therefore, that low grade infections from the field from which cultures are taken are more apt to yield growth in chicken embryos than on blood agar.

Trapnesting records indicated that infected birds not severely affected continued to lay but intermittently.

SUMMARY

Furazolidone (300 gms per ton of feed) and dihydrostreptomycin sulfate (250 mgm per bird) gave good control of infectious hepatitis in laying chickens.

Attempts to reisolate the hepatitis agent from inoculated control birds were only 40 percent successful when liver suspensions were placed in chicken embryos and 100 percent

successful when bile was used (trial 2). Bile contained the highest concentrations of the organism. The chicken embryo was the medium of choice for growing the infectious hepatitis agent.

Severely affected birds ceased to lay whereas less affected birds continued to lay but intermittently so that total production decreased.

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CONTROL OF INFECTIOUS SYNOVITIS. 8. DEGREE OF INFECTION AS INFLUENCED BY MEDICATION WITH FURAZOLIDONE AND CHLORTETRACYCLINE*

By N. O. OLSON and D. C. SHELTON

Department of Animal Husbandry and
Department of Agricultural Biochemistry and Nutrition
West Virginia University, Morgantown, West Virginia

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UNDER conditions of commerical poultry production, protection against infectious synovitis (IS) by drug medication has substantially reduced the incidence and severity of the disease. Prophylactic drug usage has been the most economical method to control IS. Contradictory reports on the efficacy of drugs against IS, merely illlustrate the variation in experimental materials and techniques used in the studies. Until more is known about the natural transmission of the disease, variable results, difficult to interpret, will be obtained with different drugs and controversy will exist as to their efficacy under field conditions.

Many conflicting reports on the efficacy of chlortetracycline (CTC) and furazolidone (nf-180) between laboratory and field trials indicate the need for some clarification and explanation of the incompatible results. Under laboratory and controlled field conditions, the difference in efficacy of these drugs appeared to be related to the amount of the inoculum of a given culture and to the virulence of the IS agent.[1-9]

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It was desired to test the hypothesis that the degree of infection would vary with the quantity of a given IS culture inoculated into the chick and the efficacy of medication would vary depending on the degree of infection. Therefore, different amounts of the IS agent were introduced into the foot pad of chicks. Different amounts of chlortetracycline and furazolidone were provided in the feed. The variation in drug efficacy was assessed according to the severity of the infection.

MATERIALS AND METHODS

Fifteen unsexed New Hampshire chicks were randomly selected, wing-banded and placed on each treatment. Chlortetracycline (CTC) and furazolidone (nf-180) were added at the rate of 50, 100 or 200 grams per ton of a commercial open formula broiler ration which contained 4 grams of procaine penicillin per ton of feed and 0.005 percent 3-nitro-4-hydroxyphenylarsonic acid. Analysis for the CTC and nf-180 content was made and the results are shown in table 1.

Infectious synovitis agent (1853) was grown in the yolk sac of six-day chicken embryos. As the infected embryos died, their yolks were harvested and stored at –60 C. Immediately prior to foot pad exposure of the experimental chicks, the frozen yolk containing the IS agent was thawed, mixed thoroughly and used as the source of the undiluted IS agent. Aliquots of this material were diluted with appropriate quantities of nutrient broth to provide dilutions of 1:5, 1:10 and 1:20 of the original concentration of the infective agent. To provide different amounts of the IS agent, 0.5, 0.25 and 0.125 ml amounts of the undiluted yolk and 0.25 ml of each dilution were used by foot pad inoculation for 18-day-old chicks. Subsequently, all birds were examined individually and weighed twice weekly during a 28 day observation period.

RESULTS AND DISCUSSION

The severity of IS in the infected control birds was greater as the amount of the virus was increased. Since relatively minor differences in pathogenicity occurred with the various amounts of the agent introduced in the undiluted yolk, a comparison of results is given in table 2 presenting the

TABLE I

Analyses of feed samples for furazolidone and chlortetracycline

Furazolidone Gm/Ton				Chlortetracycline Gm/Ton			
Sample no.	Desired content	Assayed content	Average	Sample no.	Desired content	Assayed content	Average
1	50	50.2		10	50	56	
2	50	49.2		11	50	54	
3	50	48.4	49.3	12	50	47	52
4	100	92.4		13	100	100	
5	100	88.8		14	100	87	
6	100	93.7	91.6	15	100	109	99
7	200	251.3		16	200	190	
8	200	256.6		17	200	178	
9	200	264.2	257.4	18	200	200	189

combined data for those birds receiving the undiluted yolk and those receiving the diluted yolk. The differences are expressed by showing the length of time post inoculation necessary for the birds to show visible signs of infection, the degree of swelling, the rapidity with which extension of infection to other joints takes place and the effect on weight gain. In general, with larger amounts of inoculum, the severity of the disease was greater and the incubation period was shorter.

The differences in response of the birds to the amount of inoculum and the efficacy of nf-180 or CTC are shown in figures 1 and 2. The development of signs of IS was delayed when smaller amounts of the inoculum were used. This resulted in a longer incubation period which in turn improved the efficacy of either nf-180 or CTC.

The number of infected birds, degree of swelling, number of birds in which the infection spread to joints other than those in the inoculated leg, weight gain and feed conversion are given for the control and medicated chicks in table 3.

TABLE II

A comparison of the severity and incubation period in birds inoculated via the foot pad with diluted and undiluted yolk containing the IS agent

Days after inoculation	Controls wt. gain gm.	Undiluted yolk[1] Infection[3]				Diluted yolk[2] Infection[3]			
		No.	Degree	Extension	Wt. gain gm.	No.	Degree	Extension	Wt. gain gm.
3	57	45	1.2	0	60	5	1.0	0	64
7	152	45	3.2	0	124	32	2.4	0	135
10	213	45	5.6	1	174	36	4.1	0	185
14	310	45	7.0	13	232	40	4.8	0	264
17	385	45	7.9	25	265	40	5.9	1	322
21	480	45	8.2	27	306	40	6.3	4	387
24	562	45	8.4	30	338	42	6.1	6	438
28	666	44*	8.3	33	371	42	6.6	9	500

* One died

[1] Forty-five birds inoculated via the foot pad with 0.5, 0.25 or 0.125 ml undiluted yolk containing the IS agent.

[2] Forty-five birds inoculated via the foot pad with 0.25 ml of infected yolk diluted 1:5, 1:10 or 1:20.

[3] Infection based on the following:
Number — of birds developing the disease
Degree — based on a scoring system of 0-9
Extension — number of birds showing infection in the joints other than those in the inoculated leg

TABLE III

The effect of amount of virus on the efficacy of furazolidone (nf-180) and chlortetracycline (CTC) in birds 18 days old inoculated with the infectious synovitis agent via the foot pad

Inoculum[1]	Treatment[2]		Number a	Infection[3] Degree b	Extension c	Weight gain (gm)	Feed conversion
1. Undiluted	None		15	8.8	13	286	3.29
	nf-180	50	15	6.2	5	524	2.37
		100	7	6.6	0	563	2.35
0.5 ml.		200	5	2.6	0	551	2.41
	CTC	50	5	3.4	0	573	2.41
		100	1	1.0	0	671	2.19
		200	0	0.0	0	614	2.35
	None		15	7.8	10	364	2.93
	nf-180	50	13	6.5	6	463	2.54
		100	13	4.3	0	586	2.34
0.25 ml.		200	1	1.0	0	578	2.30
	CTC	50	6	1.2	0	641	2.19
		100	0	0.0	0	615	2.40
		200	0	0.0	0	725	2.18
	None		15	8.4	9	463	2.50
	nf-180	50	13	6.2	2	563	2.39
0.125 ml.		100	11	7.3	2	568	2.37
	CTC	50	3	1.7	0	685	2.17
		100	0	0.0	0	637	2.34

II. Diluted	None		14	7.3	3	480	2.51
	nf-180	50	13	8.7	3	476	2.56
1:5 0.25 ml.		100	7	3.0	0	560	2.36
	CTC	50	2	1.0	0	625	2.26
		100	1	1.0	0	684	2.34
	None		14	5.3	3	511	2.49
	nf-180	50	8	6.7	1	550	2.21
1:10 0.25 ml.		100	8	6.6	2	535	2.35
	CTC	50	1	2.0	0	657	2.25
		100	0	0.0	0	700	2.20
	None		14	7.3	3	510	2.62
	nf-180	50	10	7.0	2	548	2.41
1:20 0.25 ml.		100	4	5.0	0	624	2.24
	CTC	50	0	0.0	0	620	2.29
		100	0	0.0	0	665	2.18
III. None			0	0.0	0	661	2.31

[1] Inoculum was yolk from infected embryos.

[2] Concentration expressed as grams per ton of feed. Fifteen birds per treatment.

[3] Severity of infection shown by the following:
(a) Number — of birds developing the disease
(b) Degree — based on scoring system of 0 to 9
(c) Extension — number of birds showing infection in joints other than those in the inoculated leg.

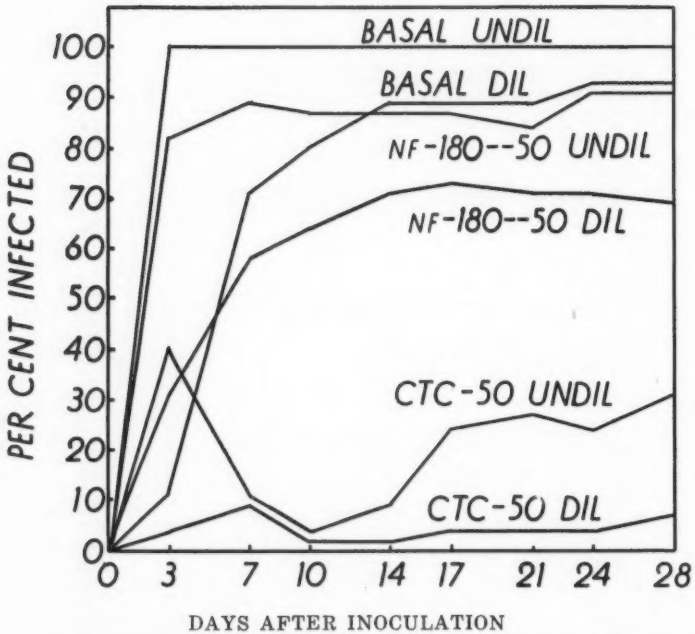

Fig. 1. A comparison of the efficacy of furazolidone (nf-180) and chlortetracycline (CTC) given at the rate of 50 grams per ton of feed when birds were inoculated via the foot pad with the diluted or undiluted infectious synovitis agent.

The less severe infection in the birds given smaller amounts of the IS agent was reflected in an increase in efficacy of both CTC and nf-180. Thus, when 0.5 ml of infected yolk was inoculated, 15/15 birds became infected and extension occurred in 5/15 birds given the 50 gram level of nf-180; whereas, 5/15 became infected and no extension occurred when the birds were given the 50 gram level of CTC. At the same levels of drug and using 0.25 ml of infected yolk diluted 1:20 10/15 nf-180 birds became infected and 2/15 birds showed extension of infection; whereas, none of the CTC fed birds became infected.

At the six levels of inoculum used, the 50 gram level of

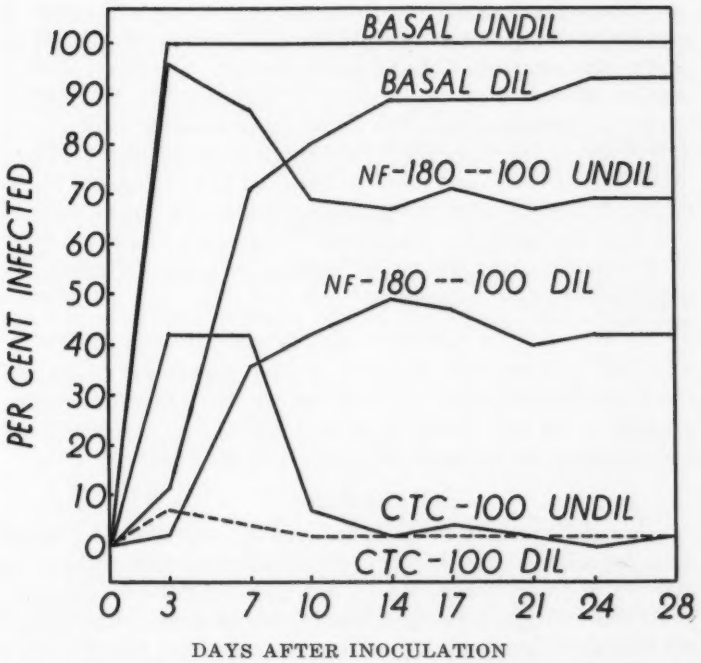

Fig. 2. A comparison of the efficacy of furazolidone (nf-180) and chlortetracycline (CTC) given at the rate of 100 grams per ton of feed when birds were inoculated via the foot pad with the diluted or undiluted infectious synovitis agent.

CTC gave consistently better results than did the 100 gram level of nf-180. The desired level of nf-180 at 200 grams per ton was 257.4 grams per ton based on feed analysis. If this level of nf-180 is compared to the 50 gram level of CTC it appears that approximately equal efficacy was obtained. However, when the undiluted yolk was administered in 0.25 ml amounts, 1/15 birds given the nf-180 at the 200 gram level became infected whereas 6/15 birds given the 50 gram level of CTC became infected. The swelling was only slight and weight gains were essentially the same. Under field conditions this would be considered a good clinical response.

These results indicate that part of the inconsistency of reports on the efficacy of nf-180 or CTC can be explained in part on the amount of the IS agent present in the bird. Where smaller amounts of IS agent are present, the incubation period is prolonged and the drugs have a longer time to act in overcoming the disease. Therefore both nf-180 and CTC are more effective against smaller doses of the IS agent. These results indicate that to compare the efficacy of drugs against the synovitis agent the drugs must be compared with the same IS inoculum. Comparisons of results between different experiments may be misleading.

ACKNOWLEDGMENTS

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SUMMARY

Six different amounts of infectious synovitis (IS) agent were inoculated into the foot pad of chickens to compare the efficacy of chlortetracycline (CTC) and furazolidone (nf-180) at 50 and 100 grams per ton. The birds receiving the two largest inoculum levels of IS were compared when treated with 200 grams nf-180 or CTC per ton of feed. As the level of inoculum was decreased, the incubation period was prolonged, the severity of the disease decreased and the efficacy of nf-180 and CTC increased.

At all comparable levels, the CTC was more effective than nf-180 in controlling IS.

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THE RELATIONSHIP BETWEEN QUAIL BRONCHITIS VIRUS AND CHICKEN EMBRYO LETHAL ORPHAN VIRUS*

By R. T. DU BOSE and L. C. GRUMBLES

Department of Veterinary Microbiology
Texas Agricultural Experiment Station
College Station, Texas

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QUAIL bronchitis virus (QBV) causes an acute, contagious, highly fatal respiratory disease in young bobwhite quail, *Colinus virginianus* (Linné). Chicken embryo lethal orphan virus (CELO) was isolated from the chicken

* A portion of the data contained in this paper was submitted to the Graduate School, Texas A & M College, by the senior author as partial fulfillment of the requirements for a Master of Science degree. R. T. DuBose now in the Department of Animal Pathology, Virginia Polytechnic Institute, Blacksburg, Virginia.

embryo. Both QBV and CELO can infect susceptible chickens and turkeys without causing a recognized disease syndrome. Quail bronchitis has been reproduced by experimental inoculation with QBV but not with CELO. QBV and CELO are related serologically. Both have been differentiated from infectious bronchitis virus (IBV) of chickens by serum neutralization (SN) tests, but not by chicken embryo response or other laboratory tests. QBV and CELO are potential contaminants of chicken embryo-propagated vaccines and may interfere with the rapid identification of IBV. Quail bronchitis is a threat to the bobwhite quail industry. The only reported significant difference between QBV and CELO is the failure of CELO to cause quail bronchitis in quail.

In this study, the relationship between QBV and CELO was determined and QBV was further characterized. A virus isolated from embryonated chicken eggs used in this work was identified as QBV (CELO). Preliminary evidence is submitted that heat stability tests may rapidly differentiate QBV (CELO) from IBV.

The isolation of quail bronchitis virus was reported in 1950 by Olson[11] and in 1957 and 1958 by DuBose, Grumbles and Flowers.[5,6] The title of Olson's report suggested "quail bronchitis" as a name for the disease. Yates, Fry and Wasserman[14] in 1954 reported the isolation of a viral agent from embryonated chicken eggs. In later studies, Yates and Fry[12,13] named this virus chicken embryo lethal orphan (CELO). In several isolations of CELO, the eggs had been inoculated with unrelated materials treated with antibiotics. It was suggested that the antibiotics used in the original inoculum may have altered the normal physiology of the infected embryo, thereby revealing the endogenous agent.[13] QBV, CELO and IBV caused a similar dwarfing and curling of the chicken embryo.[6,11,13]Cunningham has given an excellent review of the basic properties of IBV.[3]

The symptoms, course, mortality and limited gross lesions of quail bronchitis have been described in two outbreaks.[6,11] Tracheal mucus[11] and abdominal air sac exudate[6] have been

used in QBV isolations. Tracheal and lung material are commonly used for the isolation of IBV.[2] After Clark *et al.*[1] reported the isolation of Newcastle disease virus (NDV) from aqueous humor of the chicken, Flowers *et al.*[7] isolated IBV from the same source. Attempts to isolate QBV from aqueous humor have not been reported. The chorioallantoic (CA) cavity route of inoculation in 9 to 11-day-old embryonating chicken eggs has proved satisfactory for the isolation and propagation of QBV, CELO and IBV.[2,6,11,13]

No reports on the isolation or propagation of QBV, CELO or IBV in the embryonated turkey egg were found in the available literature. Delaplane[4] believed he had propagated IBV in the turkey embryo but because of unrelated circumstances was unable to complete this work.

In experimental transmission studies with QBV, Olson[11] reproduced quail bronchitis in 4-week-old quail. Coughing, sneezing and rattled breathing appeared in inoculated and contact controls. Lesions varied from none to clear or yellowish mucus in the trachea and cloudy thoracic air sacs. QBV was reisolated. When quail which had survived the disease were challenged with QBV, no symptoms developed.[6,11] Both Olson[11] and DuBose *et al.*[6] challenged chickens and turkeys with QBV. The reaction varied from none[6] to mild transitory symptoms.[11] Olson[11] reisolated the virus from both chickens and turkeys. The chickens challenged with QBV were later shown to be susceptible to IBV.[6,11]

In experimental transmission studies with CELO, Yates *et al.*[12,13,14] were unable to produce symptoms in quail, turkeys, mice, rabbits and a calf. Respiratory symptoms were not seen, but nervous symptoms appeared in young chickens inoculated intracerebrally. Chickens challenged with CELO did not lose their susceptibility to infectious bronchitis.

Serological studies showed that QBV was not neutralized by infectious bronchitis antiserum[6,11] nor by Newcastle disease antiserum.[11] Serum collected from quail which survived quail bronchitis neutralized both Olson's and DuBose's QBV.[6] Yates and Fry[13] showed that CELO virus was not serologically

related to Newcastle disease, infectious bronchitis, avian encephalomyelitis, laryngotracheitis, fowl pox and mumps viruses. Their studies did suggest a serological relationship between CELO and QBV. CELO induced a high level of specific neutralizing antibodies in the serum of inoculated rabbits.

Jungherr *et al.*[10] and Hofstad[9] reported that immunologic and antigenic differences exist in strains of IBV.

The characteristics of QBV, CELO and IBV are in general similar.[3,6,11,13] They resist antibiotics, fail to agglutinate red blood cells, increase in virulence for chicken embryos with succeeding passages, and cause similar embryo lesions. Dwarfing and curling of the embryo and thickening of the amniotic membrane are prominent changes. QBV and IBV are small enough to pass through the Seitz filter.[3,6,11] In a limited trial, the Phelps strain of CELO was retained by the Seitz filter but passed through the Berkefeld (W) candle.[13] Yates and Fry[13] reported that CELO in undiluted allantoamnionic fluid retained infectivity for the chicken embryo after being held at 56 C for 60 and 90 minutes but not for 105 minutes. Hofstad[8] tested the heat stability of 60 IBV isolates. The virus samples in chorioallantoic fluid (CAF) were diluted 10^{-2} in tryptose phosphate broth containing 20 percent heat-inactivated horse serum. Only 14 percent remained infective after 30 minutes at 56 C. Strain 189 remained infective after 45 minutes, but when diluted 10^{-2} in broth without horse serum, remained infective after 15 minutes, but not after 30 minutes at 56 C.

MATERIALS AND METHODS

The experimental procedures were designed to produce infection in quail with QBV and with CELO, to determine the symptoms and lesions in experimentally infected quail, to reisolate the viruses, to determine the specificity of resulting antibodies for QBV and CELO and to further characterize QBV.

Experimental transmission. QBV inoculations were made with strain QB 101 isolated from quail in 1956.[6] Fifteen 4-day-old quail were inoculated orally, intraocularly

and intranasally with QBV in CAF from the 5th chicken embryo passage. Intraocular and intranasal inoculations were made on one side only with approximately one drop at each site. Two days after respiratory symptoms appeared, 10 quail from the same hatch were placed in the brooder with the inoculated group. Seven days and 3 days respectively after symptoms appeared in the preceding groups, a third and fourth group of seven quail each were added to the brooder. The groups were designated QBV group I, II, III and IV, respectively.

Additional 4-day-old quail in numbers corresponding to those above were inoculated and exposed in the same manner with CELO* in CAF from the 9th embryo passage. The groups were designated CELO group I, II, III, and IV, respectively. CELO groups II, III and IV were added to the brooder 2, 4 and 0 days after the preceding group showed symptoms. The CELO groups were located 2 miles from the QBV groups.

Forty-three quail used for controls and to supply birds for QBV and CELO groups II, III and IV, were kept one-quarter mile from the CELO groups and 2 miles from the QBV groups. By necessity, young chickens, turkey poults and rabbits being raised for other work, were kept in the same building used for the control quail. Because of losses and a poor start in the control group caused by management factors, the balance of the controls was discarded 14 days after the CELO and QBV groups were inoculated. They were replaced with quail chicks of the same hatch from the same source.** There were no significant management problems at the locations housing the QBV and CELO groups.

Ten QBV inoculated or exposed quail were removed for necropsy and collection of material for virus isolation. The identity of the birds removed and the period after inoculation or exposure are shown in table 1.

Rales were noted in one control quail 25 days after inoculation of the QBV and CELO groups and 11 days after the

* Obtained from Dr. Vance J. Yates, Department of Animal Pathology, University of Rhode Island, Kingston.

** Supplied by the Texas State Quail Farm, Tyler, G. C. Stodghill, Supt.

2nd control group was received. This bird, designated control no. 1, plus 3 apparently normal control quail, designated controls no. 2, 3 and 4, were removed for observation, necropsy and virus isolation.

Two attempts were made to produce quail bronchitis with CELO at a time when no transmission trials were being conducted with QBV. To preclude contamination of the inoculum with QBV, CELO in CAF was produced by using for inoculum 6th chicken embryo passage material obtained directly from Dr. Yates. Inoculation of embryonating eggs and harvesting of CAF was carried out on days when work had not been done in the laboratory with other agents.

In the first attempt, 10 quail used as controls for the preceding work were placed in isolation at 46 days of age and designated CELO group V. Six were inoculated orally, intranasally and intraocularly with CELO in CAF. Four served as contact controls. They were observed for 20 days. Necropsies were not made nor were virus isolations attempted.

For the second attempt, ten 3-week-old quail were obtained from a privately owned flock with no history of respiratory symptoms and were designated CELO group VI. Five were inoculated intranasally and intraocularly with the same material used for CELO group V. Five controls were placed in a separate brooder in the same room. On the 7th and the 12th day after inoculation, one inoculated and one control bird were killed to obtain material for virus isolation atempts. Necropsies were not preformed.

Reisolation of virus. With the exception of CELO group V, two or more birds from each inoculated or exposed group of quail were used for virus isolation attempts. Material was collected from the anterior chamber of the eye, from the trachea and from any other organ showing significant lesions. The brain was collected from two quail with nervous symptoms. In some instances, material was pooled from the same organs of several birds of the same group. Bird identity, period after inoculation or exposure and organs used for the isolation atempts are shown in table 1 for QBV and CELO groups I through IV. Aqueous humor and tracheal material

was collected 7 days and 12 days after inoculation of CELO group VI from one inoculated and one control bird for each period.

Aqueous humor was collected by inserting a 24-gauge needle near the corneo-scleral junction into the anterior chamber and aspirating the fluid into a one ml tuberculin syringe. The method of collecting aqueous humor from CELO group IV, birds 1, 2, 3 and 4, was modified in order to determine whether virus isolations which were apparently from aqueous humor, could actually result from virus laden lacrimal fluid entering or coating the syringe needle. One eye of each bird was enucleated, immersed in 50 percent ethyl alcohol for 10 minutes, rinsed in 3 changes of sterile distilled water and the aqueous humor then aspirated. Aqueous humor was aspirated from the other eye *in situ* and without alcohol treatment.

Except for aqueous humor, the collected material was routinely ground by mortar and pestle and suspended in nutrient broth (Difco). All material was treated with 25 mg dihydrostreptomycin sulfate and 1,000 units potassium penicillin G per ml of suspension for 30 to 40 minutes at room temperature. The suspensions and harvested CAF were stored at 0 C to –20 C until embryonated chicken eggs were available. Organs showing lesions suggestive of bacterial infection were cultured on tryptose or blood agar. Isolation of PPLO in carbohydrate medium plus serum fraction was attempted from the respiratory tracts of six quail.

Veterinary Research Farm (VRF) eggs from an unvaccinated flock of chickens with no history of respiratory disease were used to supply chicken embryos for virus isolations during the first and major part of this study. The VRF flock was housed approximately 50 yards from the control quail. College Poultry Farm (CPF) eggs from a flock of chickens which had been vaccinated for Newcastle disease and infectious bronchitis were used toward the last part of the study.

Nine to ten-day-old chicken embryos were inoculated via the CA cavity with the suspended material or with CAF. Inoculated and control embryos were candled at least once

daily. Those dying within 24 hours after inoculation were discarded. CAF was harvested at the time embryos showed dwarfing but routinely no later than the 6th day after inoculation. Dwarfing observed by candling was graded as suspicious, slight, moderate and severe. Moderate dwarfing of at least two, plus slight dwarfing of at least one out of five inoculated embryos was the criterion for determining the successful isolation passage (table 1). At least three blind passages were made before an attempt was judged negative.

During the period when VRF eggs exclusively were used for virus isolation attempts, 23 14-day-old VRF embryonated eggs were candled preparatory to necropsy and study of normal organs. Six of the embryonated eggs showed a slight excess of CAF. The eggs were paired, CAF harvested and pooled from each pair, and the harvests labelled controls A, B, and C, respectively. No antibiotics were used. The CAF was passaged in chicken embryos in the same manner used for material collected from quail. Two weeks later another VRF embryonated control egg showed a slight excess of CAF at 15 days of incubation. CAF was harvested from this egg, designated control D and passaged in chicken embryos.

Serology. Comparative serum neutralization tests were made to determine the ability of CELO antiserum (rabbit)* to neutralize CELO* and QBV. In general the method for serum neutralization described by Cunningham[2] was followed except that the serum was diluted 10^{-1} in PSS. Serum was collected from each group of inoculated or exposed quail at periods ranging from 13 to 73 days postinoculation (PI) or postexposure (PE). The PI or PE periods for the QBV groups averaged 61.5 days and for the CELO groups 25.5 days. Equal quantities of serum from each inoculated or exposed group were pooled for use as QBV or CELO antiserum (quail) respectively. Serum was collected and pooled also from control quail 2, 3, and 4.

Serums from the QBV groups, from the CELO groups and from the controls 2, 3, and 4, were diluted 10^{-1} in nutrient

* Obtained from Dr. Vance J. Yates, Department of Animal Pathology, University of Rhode Island, Kingston.

broth and tested for their respective abilities to neutralize QBV and CELO virus.

CELO antiserum (rabbit) diluted 10^{-1} in nutrient broth was tested for its ability to neutralize a virus, designated control B virus, which had been isolated from the VRF eggs.

Characterization. To characterize QBV and in an effort to find a relatively simple method of differentiating QBV, CELO and IBV, studies were made on chicken and turkey embryo response to the viruses, effect of filtration and effect of heat treatment. In addition, QBV was tested for its ability to resist sedimentation by centrifugation.

Observations were made on the chicken embryo mortality pattern, dwarfing and the gross lesions of necropsied embryos in the reisolations of QBV and CELO, the filtration of CELO, in straight passages (9th through 11th) of two field strains of IBV designated Van (Massachusetts strain)* and Draper, in the serum neutralization test on control B, and in heat tests on control B virus and Haralson IBV (tentative identification).

Ten-day-old to 14-day-old embryonated turkey eggs were inoculated via the CA cavity with QBV in CAF from the 5th chicken embryo passage. CAF from dwarfed embryos or embryos that died was used for inoculation of turkey embryos in succeeding passages. CAF harvested from each passage in turkey embryos was also inoculated into 9 to 11 day-old chicken embryos. At least two embryos from each passage were necropsied and the gross lesions recorded. Periodically, harvested CAF was cultured for pleuropneumonia-like organisms in carbohydrate medium plus serum fraction. Attempts to propagate CELO in CAF from the 7th chicken embryo passage and Van IBV in CAF from the 7th chicken embryo passage were made in turkey embryos in the same manner.

The ability of QBV to pass through the Seitz filter has been reported.[6,11] Passage of IBV through the Seitz filter is a routine measure for the elimination of bacterial contaminants. To confirm previous work on QBV and to de-

* Obtained from Dr. Henry Van Roekel, University of Massachusetts, Amherst.

termine filtration characteristics of CELO, QBV in CAF from the 5th chicken embryo passage and CELO in CAF from the 7th chicken embryo passage were subjected to filtration tests using the Seitz EK disc in a Swinney filter adapter. The test was repeated on the CELO virus. In addition, QBV in CAF from the 10th embryo passage was tested for its ability to pass through the Selas 03 and the Selas 02 filters, subjecting the virus material to an unmeasured vacuum for 80 minutes and 20 minutes respectively. Cunningham[2] listed the maximum pore sizes for these filters as 0.6 micron (Selas 03) and 0.85 micron (Selas 02).

QBV in 30 ml of CAF from the 10th chicken embryo passage was placed in a 50 ml centrifuge tube and centrifuged at 10,000 rpm for 20 minutes in a Lourdes Model AT, high speed, angle-head centrifuge. While *in situ*, the centrifuged CAF was very slowly removed from the top downward in 5 ml portions, using 10 ml syringes equipped with 1 1/2 inch or 2 inch, 16 gauge needles. The first, third and last portions were used for embryonated egg inoculations. For a second test, the centrifuge was placed in a room maintained at 4 to 5 C and allowed to chill before use. Three and one-half ml of a pool of the first and second portions (uppermost third) of the material subjected to the 10,000 rpm, 20 minute test was used for the second test. When placed in a small centrifuge tube, the fluid made a column approximately 6 cm high. This material and a tube of water were centrifuged 30 minutes at 15,000 rpm (28.7 x g). The CAF was then removed in the same manner but in one ml portions, each portion accounting for approximately 2 cm height of the column of liquid. At the end of the centrifugation period, the water had a temperature of 38 C. Each portion of the CAF from this test, the designated portions of the previous test, and the original uncentrifuged material were used for inoculation of 10 and 11 day-old embryos in 0.2 ml amounts via the CA cavity.

For a limited study on heat stability, the following viruses

* Connecticut IBV No. A5968, obtained from R. E. Luginbuhl, Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

in CAF from the chicken embryo passage noted were used: QB-4P, CELO-7P, Van IB-3P, Draper IB-8P, Conn IB*-13P, control B-3P and Haralson IB (tentative identification)-2P and 4P. The Haralson virus was recently isolated from the tracheas of chickens submitted to the diagnostic laboratory. It was identified as IBV on the basis of dwarfing produced in chicken embryos and negative hemagglutination tests. Virus materials were sealed in 10 ml Becton-Dickson vacuum tubes and immersed in a water bath at 56 C $\pm$ 1 C for the time indicated. In the preliminary trials in which undiluted CAF was used, the tubes were not submerged but their fluid level was at least 2.5 cm below the level of the water bath. In trials with diluted CAF, the tubes were completely immersed with the water bath level at least 2.5 cm above the tubes. No agitator was used. Two minutes were allowed for heating of the tubes before timing started on the indicated heating periods.

CELO in undiluted CAF was treated for 30, 60 and 105 minutes at 56 C. Haralson virus (2P) in both undiluted CAF and dilutions of 10^{-3} in nutrient broth was held at 56 C for periods of 45 minutes and 60 minutes. QBV, CELO, Haralson and control B viruses in CAF diluted 10^{-2} in nutrient broth were held at 56 C for periods of 30, 60 and 90 minutes. The Van, Draper and Connecticut (A5968) strains of IBV in the same dilution were held at the same temperature for 30 and 60 minutes. Viability of the viruses was tested by chicken embryo response to inoculation with the heat-treated CAF. Non-heated portions of each virus material were tested in the same manner.

RESULTS

Symptoms. Respiratory symptoms appeared in QBV group I and CELO group I quail 4 days and 3 days postinoculation, respectively. They appeared in QBV group II and group III 2 days and 7 days after contact exposure, respectively. No symptoms appeared in QBV group IV. Respiratory symptoms appeared in CELO group II, group III and group IV 5, 9 and 2 days after contact exposure, respectively. Symptoms in CELO group IV were mild and transitory, lasting no more

than one day. Incubation periods are shown in table 2 for QBV and CELO groups I through IV.

TABLE I

Virus isolations from experimental quail

Group	Bird number	Days PI-PE	Aqueous Humor	Trachea	Other organs
QBV					
II	1	7	1P	1P	Lu-1P; Br-1P
II	2	7	1P	1P	
II	3	11	1P	1P	Tas-1P; Lu-1P
II	4	11	1P	neg.	Aas-1P
I	1	13	1P	1P	Sp-1P
I	2	14	neg.	3P	
IV	1,2,3,4*	48	2P	3P	
CELO					
I	1	12	1P	3P	Lu-3P
I	2	12	1P	3P	Br-3P
IV	1,2,3,4*	13	3P	2P	Cj-2P
II	1,2*	16	3P	5P	
III	1,2,3,4*	22	2P	neg.	
II	3,4,5*	31	3P	neg.	
I	3,4,5,6*	36	neg.	neg.	

* - Material from same organs pooled for virus isolation.
PI - Postinoculation.
PE - Postexposure.
P - Embyro passage in which dwarfing was definite.
neg. - isolation attempt negative.
Organs:
Aas - Abdominal air sac, Br - Brain, Cj - Conjunctiva
Lu - Lung, Sp - Spleen, Tas - Thoracic air sac.

TABLE II

Incubation periods in days

Virus	Quail groups			
	I	II	III	IV
QBV	4	2	7	ns
CELO	3	5	9	2?

ns - No symptoms noted.
? - Questionable or transitory.

The first symptom shown in QBV group I (inoculated) and CELO group I (inoculated) was difficulty in breathing, the mouth being opened and closed during each respiratory cycle. The symptom was not shown when the quail were

moving about nor when their attention was directed toward the observer. When the observer remained still for several minutes the symptom appeared in those quail chicks not engaged in eating or in moving about.

The progression of respiratory symptoms was similar in QBV group I and CELO group I. Oral respiration, bellowing in and out of the skin over the infraorbital sinus, sneezing, slinging of the head, swelling of the sinus and conjunctivitis appeared approximately in that order. The sound of sneezing in young quail was very low in volume and was easily masked by extraneous noises. It was best observed at night when the quail were huddled and asleep. Suddenly a quail chick would thrust its head upward and forward, extend its neck, open its mouth, and emit the soft sound of the sneeze. Occasionally 2 or 3 distinct sneezing sounds would be made with one extension of the neck. Sneezing by one bird seemed to initiate the reflex in several birds. The "popping up" of heads and necks above the surrounding level of huddled backs was distinctive. The sneezing session would be followed by an interval of quiet in the group as a whole.

Noticeable swelling of the sinus first appeared 8 days after inoculation in the QBV and CELO group I. It was usually unilateral and usually, but not always, on the side of the intraocular inoculation. Nasal discharge could not be discerned while the quail were in the brooder.

Respiratory symptoms in QBV group II followed the same order as in group I except that bellowing of the sinus was observed before oral respiration. In CELO group II, respiratory symptoms were less marked than in QBV group II. In QBV group III only oral respiration appeared in one bird and conjunctivitis in another. No symptoms were presented by QBV group IV and only transitory oral respiration appeared in CELO group IV.

The progression of respiratory symptoms noted above applies to the groups. The high level of activity in young quail and the difficulty in identifying individual birds without handling them precluded recording progressive symptoms in individual quail.

Nervous symptoms were observed in two quail. In QBV group II one bird became affected 7 days after exposure. It would suddenly stand upright for 3 or 4 seconds, then fall over backward. This would be repeated several times, then no symptoms would be shown for intervals of from 5 to 15 minutes. Disturbing the bird would initiate the symptoms. Occasionally the head was placed between the feet and 2 or 3 successive forward somersaults were executed. Respiratory symptoms were not observed. This quail was killed on the day the symptoms were noted and designated QBV group II, bird 1.

In CELO group I, 3 days after inoculation, one bird, designated CELO group I, bird 1, was observed to assume an unnatural upright position with the axis of the body and neck almost vertical and the beak pointed downward. The symptoms remained consistent for 9 days, at which time the bird was killed.

CELO group V, obtained from the pool of control birds and inoculated at 46 days of age, showed no symptoms during the 20-day observation period.

CELO group VI, obtained from a privately owned flock and inoculated with CELO when no work was being done with QBV, showed respiratory symptoms 3 days after inoculation. Both inoculated birds and controls showed oral respiration and bellowing at the sinus. Symptoms disappeared in 8 to 11 days.

Lesions. One QBV-inoculated quail, designated QBV group I, bird 1, died 12 days after inoculation. The eyelids were pasted together over both eyes and the left (uninoculated) conjunctival cavity, lacrimal canal and sinus were packed with caseous exudate. The right (inoculated) conjunctival cavity contained mucoid exudate. The thoracic air sac contained a small amount of cream-colored mucoid exudate. The lungs were edematous. Postmortem decomposition was general in the balance of the viscera. Bacteria tentatively identified as *Alcaligenes sp.* were isolated from the sinus and spleen. Attempts to culture PPLO from the sinus and trachea were negative.

One QBV-exposed bird, designated QBV group II, bird 3, was moribund 11 days after exposure. The right eyelids were adhered, the right sinus and lacrimal canal packed with caseous exudate, the thoracic air sac contained mucoid to caseous exudate, the anterior abdominal air sac contained foamy mucoid exudate and the spleen presented a granular appearance. A slight amount of mucus was noted in the trachea. Material from the trachea, liver and air sacs separately cultured on blood agar yielded the organisms tentatively identified as *Alcaligenes sp.* PPLO could not be isolated from the trachea, sinus and air sacs.

No visible lesions were found in CELO group I, bird 2, which had exhibited nervous symptoms. The other quail with nervous symptoms, QBV group II, bird 1, showed a slight paleness of the spleen and kidney.

Control bird 1 which exhibited rales was killed and necropsied 2 days after symptoms were observed. No visible lesions were observed. Controls 2, 3, and 4 which had been observed in contact with 1 for 2 days, then observed in a separate coop for 7 additional days, developed no symptoms. No gross lesions were noted.

The lesions observed in the balance of the necropsied quail in groups I through IV inoculated with or exposed to QBV or CELO are given in table 3. The food coloring used on the feathers of QBV group III for identification had faded to such an extent that quail of that group could not be accurately identified. For this reason quail from QBV group III were not killed for necropsy nor virus isolation done 30 to 40 days postexposure as planned.

Reisolations. The results of attempts to reisolate the viruses from QBV and CELO inoculated or exposed quail in groups I through IV are shown in table 1. Within the QBV and CELO groups, the birds are listed in order of time between inoculation or exposure and the attempt to reisolate the virus. QBV was reisolated from QBV group IV, birds 1, 2, 3, and 4, 48 days postexposure.

With only one exception, when virus was isolated from the trachea, it was also isolated from the aqueous humor. There

TABLE III

Lesions in QBV and CELO quail

		PI-PE	Eye	Trachea	Sinus	Lung	Tas	Aas	Liver	Spleen	Kidney
QBV											
I	1 *	13	caseous, conj.	—	caseous	edema	mucus	—	—	—	—
	2	14	conj.	—	caseous	—	—	mucus	—	pale	pale
II	1 *	7	—	—	—	—	—	—	—	pale	pale
	2	7	—	—	—	—	—	—	pale, patches	pale	pale, urates
	3 *	11	conj.	?	caseous	—	mucus, caseous	mucus	—	granular	pale
	4	11	—	—	—	—	cloudy	mucus	—	granular	—
IV	1-4 incl.	48	—	—	—	—	—	—	—	—	—
CELO											
I	1	12	conj.	—	—	—	—	—	—	—	—
	2	12	—	—	—	—	—	—	—	—	—
II	1	16	—	—	—	—	—	—	—	—	—
	2	16	—	—	—	—	—	—	—	—	—
II	3-5 incl.	31	—	—	—	—	—	—	—	—	—
III	1-4 incl.	22	—	—	—	—	—	—	—	—	—
IV	1-4	13	—	—	—	—	—	—	—	—	—

PI - Days postinoculation, PE - Days postexposure TAS - Thoracic air sac, Aaas - Abdominal air sac
conj. - conjunctivitis, * - Lesions discussed in text

were 3 instances in which isolation from the trachea failed, but virus was recovered from the aqueous humor.

In the test on material from CELO group IV, birds 1, 2, 3, and 4, to determine whether the virus was actually in the aqueous humor, the virus was recovered in aqueous humor from the alcohol-treated eyes, from the untreated eyes and from the conjunctival swabbings. Presence of the virus in aqueous humor was verified.

QBV or CELO was isolated from the aqueous humor and the trachea—air sac—lung pool of control 1 in which rales had been noted. QBV or CELO was isolated from the pooled aqueous humor of controls 2, 3, and 4, but not from the pooled tracheal material.

CELO was reisolated from CELO group VI quail which has been experimentally infected when no work was being done with QBV. Both aqueous humor and tracheal material collected 7 days and 12 days after exposure from inoculated and from control birds caused moderate to severe dwarfing in embryonating chicken eggs in the first passage.

Untreated CAF, designated control B and C, respectively from VRF eggs set for incubation 8 days after inoculation of the CELO and QBV Group I quail, caused suspicious or slight dwarfing in the first chicken embryo passage. In the 2nd passage inoculation with both antibiotic treated and untreated CAF caused severe dwarfing and death of all embryos by the 3rd day after inoculation. Controls A and D were carried through 4 blind passages before being judged negative. Attempts to isolate PPLO and other bacterial agents from the original and succeeding inoculums failed.

Serology. In the comparative study of neutralization of known QBV and Yates' CELO virus, Yates' CELO antiserum (rabbit) diluted 10^{-1} in nutrient broth protected more than 50 percent of embryos inoculated with undiluted QBV which titered 6.6 based on e.l.d.$_{50}$ in 6 days. The same diluted CELO antiserum protected more than 50 percent of embryos inoculated with undiluted CELO virus which titered 6.4 based on e.l.d.$_{50}$ in 6 days. This corresponds with the finding of Yates and Fry[13] that CELO antiserum neutralized significant

amounts of Olson's[11] QBV.

The results of neutralization tests on QBV and CELO with sera collected from control quail, from QBV quail and from CELO quail are shown in table 4. The indices of neutralization against both viruses differed little for each antiserum, respectively. The difference between indices of the 3 antisera against the same virus may be insignificant. On the other hand, the greater neutralizing ability of QB antiserum may reflect the 61.5 day average PI-PE period prior to serum collection as compared to the shorter 25.5 day average PI-PE period for serum from the CELO quail. Antiserum from the control quail neutralized CELO, adding to the evidence that the controls were unintentionally infected with CELO.

Yates' CELO antiserum (rabbit) diluted 10^{-1} in PSS completely protected embryos inoculated with control B virus. This adds to the evidence that control B virus, endogenous to VRF embryonated eggs, is CELO or QBV. Control C virus was not tested by serum neutralization.

TABLE IV

Serum neutralization indices*

Virus	CELO quail Antiserum	QBV quail Antiserum	Control quail Antiserum§
CELO†	3.8	4.8	4.1
QBV‡	3.9	5.0	—

* - Indices adjusted to correct for 10^{-1} dilution of quail serum in nutrient broth.

† - CELO virus, 7th chicken embryo passage, with e.l.d.$_{50}$ titer of 4.3 at 4 days postinoculation.

‡ - QBV, 4th chicken embryo passage, with e.l.d.$_{50}$ titer of 4.4 at 6 days postinoculation.

§ - From control quail 2, 3, and 4 after unintentional infection.

Characterization. No significant differences were found in the mortality pattern, dwarfing or gross lesions of chicken embryos inoculated with Van IB, Draper IB, CELO, QB, control B or Haralson virus.

QBV was carried through 4 passages in turkey embryos. Two out of four embryos died in the first passage on the 9th and 13th day after inoculation, respectively. Of two embryos

in the second passage, one died on the 9th day and one on the 12th day PI. Of three embryos in the third passage, one died on the 13th day. Of five embryos in the fourth passage, one died on the 12th day. Two extra turkey embryos were included in each of the passages for chilling between the 4th and 7th day and harvesting CAF as inoculum for the succeeding passage. Embryos which died were examined for gross lesions. Those from the first and second passage presented lesions similar to those seen in chicken embryos inoculated with QBV or CELO. In the third passage only one embryo had lesions, a slight thickening of the amnionic membrane and slight engulfment by the yolk sac. Dwarfing was questionable in the first passage, severe in one embryo in the second passage, and absent in the third and fourth passages. Under the limited conditions of this trial, pathogenicity of QBV seemed to decrease for the turkey embryo, yet the CAF harvested from successive passages in the turkey embryo produced increasing signs of virulence for the chicken embryo. The average PI day of death for chicken embryos inoculated with CAF from successive passages of QBV in the turkey embryo were 7 days, 7.6 days, 4.3 days and 4 days.

CELO was propagated in turkey embryos through three passages. The response resembled that seen in chicken embryos. Virulence to the turkey embryo did not seem to decrease as it did with QBV.

Van IBV (Massachusetts strain) was propagated in turkey embryos through four passages. Dwarfing and deaths were erratic. Van IBV in CAF from the fourth turkey embryo passage produced a typical reaction in chicken embryos. Propagation in turkey embryos was not found to be a method of differentiating QBV, CELO and IBV.

In the filtration studies, QBV and CELO were passed through the Seitz filter (EK). QBV was passed through the Selas 03 and Selas 02 filters. The respective filtrates produced a response typical for the virus when inoculated into embryonated chicken eggs.

QBV was present in the upper 5 ml portion of a 30 ml quantity of CAF after centrifugation at 10,000 rpm for 10

minutes, and also present in the upper 2 cm of a 6 cm column of CAF after centrifugation at 15,00 rpm for 30 minutes.

In the stability tests, CELO in undiluted CAF remained viable after being held at 56 C for 105 minutes. Haralson virus in undiluted CAF from the 2nd chicken embryo passage withstood 60 minutes and in CAF diluted 10^{-3} in nutrient broth withstood 45 minutes at 56 C. After these preliminary tests, dilutions of 10^{-2} were used to gain a closer comparison with Hofstad's report[8] on the stability of IBV at 56 C. The results are shown in table 5. Neither Van, Draper nor Conn IBV remained viable after 30 minutes at 56 C. QBV, CELO, control B virus and Haralson "IBV" remained viable after 90 minutes. Although serum neutralization and chicken infectivity studies should be made before final conclusions are drawn, it seems possible that Haralson "IBV" is not IBV, but QBV (CELO) instead. Repassage of the original Haralson tracheal suspension in CPF embryonated eggs 49 days after the initial passage, produced typical dwarfing and mortality. This eliminated the possibility that Haralson virus was endogenous to an embryo of the initial passage.

TABLE V

Heat stability tests

Virus*	No Heat	Time in minutes at 56 C		
	Control	30	60	90
QBV	+	+	+	+
CELO	+	+	+	+
Van IBV	+	—	—	nt
Draper IBV	+	—	—	nt
Conn IBV	+	—	—	nt
Har IBV†	+	+	+	+
Control B	+	+	+	+

+ - Viable after treatment.
— - Inactivated.
* - Virus in chorioallantoic fluid diluted 10^{-2} in nutrient broth
† - Haralson virus, initially identified as IBV.
nt - No test at this period of time.

DISCUSSION

Symptoms in relatively mild forms of quail bronchitis may not be noticed and immunity may develop without detection of the infection. Infection of the control quail in this study

is attested by appearance of symptoms in one bird, isolation of the virus from that bird and from others not showing symptoms and by lack of susceptibility of 10 of the controls (CELO group V) when challenged with CELO at 46 days of age. The same challenge material produced the disease in quail from another flock.

In spite of high mortality in reported field outbreaks,[6,11] neither Olson[11] nor the authors of this report have been able to demonstrate mortality of any consequence in experimentally induced quail bronchitis. QBV may rapidly lose pathogenicity for quail when propagated in the chicken embryo. Information on the relative changes in pathogenicity and immunogenicity may be of value for QBV propagated in chicken embryos and for QBV and IBV propagated in turkey embryos.

Accidental infection of the control quail caused some difficulty in interpreting data on experimental transmission of quail bronchitis. Development of symptoms in both the QBV and CELO groups within 2 to 9 days after inoculation or exposure by contact (table 2) supports the belief that the disease resulted from experimentally induced infection of these groups.

The validity of some of the virus isolations from QBV and CELO groups I through IV could be questioned on the basis of CELO having been isolated from uninoculated embryonating eggs from the veterinary research farm. The reisolations as a whole are supported by the fact that changing the source of egg supply had no effect on the uniform success of reisolation attempts.

It is believed that CELO was transmitted from the CELO group I quail to the control quail and to the VRF chickens by air (one-quarter mile), on the clothing of attendants or by wild quail which have been seen on the farm. If the chickens were infected by CELO used for transmission trials in quail, then transmission of the virus through the egg must begin to occur within 8 days after infection. VRF eggs from which endogenous CELO was isolated were laid within 2 to 8 days after the quail were inoculated.

The use of aqueous humor as a source material by Clark *et*

al.[1] for NDV, by Flowers *et al.* for IBV[7] and in this study for QBV (CELO) should emphasize its probable value in the isolation of avian respiratory viruses. If the results shown in table 1 are confirmed by further work in isolations from natural outbreaks, aqueous humor should supply the virus more often than the trachea and should give earlier evidence of the isolation in chicken embryo passages.

Isolation of QBV (CELO) from the brains of two quail showing nervous symptoms supports evidence presented by Olson[11] and by Yates *et al.*[13] that the agent sometimes invades the central nervous system.

The comparative lack of lesions (table 3) in the CELO inoculated and exposed groups is not explained. The CELO inoculum (9th passage) might have been more embryo-adapted and less pathogenic for quail than the QBV inoculum (fifth passage), but this is not supported by any marked difference in type or degree of symptoms produced. Although further identification might be of value, the bacteria tentatively identified as *Alcaligenes sp.* were probably nonpathogenic secondary invaders.

The significance of pale spleens and kidneys (table 3) is questionable because sufficient control quail were not available for comparison. Gross lesions pathognomic for quail bronchitis were not demonstrated.

QBV and CELO survived 90 minutes at 56 C and three known strains of IBV were inactivated by 30 minutes at 56 C. Heat stability tests may provide a method for rapid differentiation of QBV and IBV. Further trials with different periods of heat treatment and with additional strains of QBV and IBV are being conducted.

QBV and CELO could not be differentiated by methods used in this investigation. Development of respiratory symptoms in quail infected with CELO provides further evidence that QBV and CELO are the same agent.

SUMMARY

1. Quail bronchitis virus (QBV) and chicken embryo lethal orphan virus (CELO) produced a similar disease in bobwhite

quail. Contact transmission was demonstrated with both agents.

2. Mortality pattern, dwarfing and gross lesions in chicken embryos caused by QBV could not be differentiated from that caused by CELO.

3. Reciprocal serum neutralization studies demonstrated no antigenic differences between QBV and CELO.

4. Aqueous humor was equal or superior to tracheal material as a source for isolation of QBV and CELO.

5. CELO was isolated from uninoculated chicken embryos. Antibiotics were not necessary to unmask the virus.

6. QBV, CELO and IBV were propagated for a limited number of passages in turkey embryos but could not thereby be differentiated.

7. QBV and CELO survived 90 minutes at 56 C. Three known strains of infectious bronchitis virus (IBV) were inactivated by 30 minutes at 56 C. Heat stability may be a rapid method of differentiating QBV (CELO) and IBV.

8. QBV and CELO could not be differentiated by any of the methods used in this study. They are considered to be the same agent.

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CASE REPORT—BOTULISM TYPE C OUTBREAK ON A PHEASANT GAME FARM

By SRIKRISHNA VADLAMUDI,* V. H. LEE, and R. P. HANSON

Departments of Veterinary Science and Bacteriology
University of Wisconsin, Madison, Wisconsin

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A DISEASE with heavy mortality occurred among ring-neck pheasants on a game farm in northeastern Wisconsin in the summer of 1958. Sickness and deaths among the birds were first noticed on July 19 and within 3 days about 400 birds died. The disease was confined to two adjoining pens. Treatment administered by the farm manager consisted of Sulmet† and terramycin given in the drinking water.

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* On leave from the Animal Husbandry Department, Andhra State, India.

† Sulmet(R) brand of sulfamethazine, American Cyanamid Company.

There were no sick birds at the time the authors visited 9 days after the onset of the disease. Twenty-six blood samples and other clinical materials and insects were collected on this visit.

From the last week of July until September 10, other pens were infected and about 10,000 pheasants or 15 percent of the total flock died. All except six of the 100 pens became involved.

Mortality occurred in the form of successive waves. Four or five birds would die in a pen on the first day. On each of the 2nd and 3rd days, up to 30 birds would be found dead in the pen. The mortality then would taper off to a few or no deaths, only to return in the same pen a week or more later.

Farm management. The game farm consists of 625 acres of grassland near Crivitz in Marinette County on which about 65,000 pheasants have been raised yearly since 1946. Approximately 5,000 hens are retained each year for breeding. The cocks are placed with them only during mating season. The incubator facilities and the fully equipped brooding houses are well separated from the rearing pens. The birds are moved from brooder houses to field runs or rearing pens when they are about 6 weeks old. About 400 birds are placed in each of the pens which are one acre or larger. Runways around the periphery of the pens are cut in grass that otherwise forms a cover for the birds in the interior of the pen. The earth was ploughed when the farm first began operation. Since that time, the soil has not been disturbed or disinfection attempted. Wire mesh is used for fencing and stands about 7 feet high so that birds may occasionally, when disturbed, fly over it into adjacent pens. All the rearing pens are located on high-lying sandy land well separated from the marshland hunting grounds maintained by the farm. Surrounding the entire establishment is a mixed conifer-hardwood forest. Small pools are common on the hunting grounds and a river runs near the farm.

The feed is obtained from two commercial firms. The birds receive the mash twice a day in feed troughs placed in

the pens. Water from a farm well is transported in tanks to the pens. An automatic float system provides the birds a continuous supply of water from a barrel in each pen.

The pens are not covered and wild birds have free access. Crows and starlings are known to feed and drink with the pheasants. Carcasses of wild birds are found in the field runs occasionally. The carcasses of pheasants are generally removed from the pens but thorough searches are not made except during times of heavy mortality when attendants walk through the pens regularly to search for and remove sick and dead birds.

Eight species of mosquitoes and one species of biting midge were obtained in light traps and pheasant-baited traps operated for 2 days, July 28 and 29. A species of mosquito (*Culiseta morsitans*) and a species of *Culicoides* (*C. crepuscularis*) which apparently had fed upon the pheasant "bait" were recovered from the baited traps. Filth-breeding flies *(Calliphoridae)* were present around the feeding areas and on carcasses of dead birds.

DESCRIPTION OF THE DISEASE

The onset of disease in the pheasants was sudden with few observed signs of illness. Affected birds were first seen to drag their feet. Diarrhea occurred in some cases. The birds became prostrate and death resulted in about 3 hours. A loss or loosing of feathers was associated with death.

During a second visit to the farm on September 10, several dead birds were necropsied on the premises and some were later examined at the Animal Disease Diagnostic Laboratory at Madison. All six birds examined on the farm had crops and gizzards full of feed. One individual had an inflamed intestine. There was, in general, a large amount of mucus in the intestines. In one case, the spleen was enlarged to about three times normal size and was fragile. No other abnormality was noticed. Liver, spleen, blood, brain and intestines were collected for isolation of an etiological agent.

One sick and prostrate bird was found whose eye was injured and infested with fly maggots *(Calliphoridae)*. Apparently blowflies were attacking any site of injury.

Seven sick birds, which were given polyvalent *Cl. botulinum* antitoxin Types A, B, and C on September 10, recovered. About 20 sick birds were removed from the farm to other premises by the owner and recovered without any treatment.

Diagnostic studies. Helminthic parasites were seen in several of the birds autopsied. In two birds, amoeboid stages of *Histomonas meleagridis* was found in the cecal content. In one bird autopsied in the Animal Diagnostic Laboratory, *Heterakis gallinae* eggs were found in intestinal content.

The liver, spleen and intestinal materials of dead and autopsied birds were cultured in thioglycolate broth, nutrient agar and tetrathionate broth. Gram-positive rods and spores resembling *Clostridia* were found in stained preparations. Gram-positive cocci and gram-negative rods were also observed.

Virus isolations in chicken embryos were attempted from liver, spleen and brain tissues of 10 birds autopsied. No neurotropic or viscerotropic virus was detected. Twenty-four serums from birds on the farm were tested for the presence of antibodies against eastern equine encephalomyelitis, western equine encephalomyelitis and Newcastle disease. Antibodies for these viruses were not detected.

Two crows. one dead and one partially paralyzed, were found in the pens. Virus isolation attempts from these birds were negative. No cultures for bacteria were made from the crows.

The spleen of one bird obtained September 10 was fragile, enlarged about three times its normal size and had white spots on the surface. Microscopic examination of the spleen and liver revealed necrotic patches of glandular tissue with toxic degeneration of the surrounding cells. One gram quantities of each of the intestines, liver and spleen of two birds were ground in 5 ml of normal saline and inoculated subcutaneously, 0.5 ml each, into 6 adult mice. All the mice died within 24 hours with typical signs of botulism: respiratory distress, paralysis and death. Three other samples of the liver and spleen from typically affected birds were not lethal to mice.

Cl. botulinum Type C antitoxin, diluted 1:2, was mixed in equal quantities with liver and spleen supernatant fluid and

0.5 ml of the mixture was inoculated subcutaneously into each of six mice. Six other mice serving as controls were each given 0.5 ml of the supernatant fluid subcutaneously. All of the six controls died within 36 hours; whereas, the mice injected with antitoxin-treated material were unaffected.

Two ml of the toxic supernatant was injected subcutaneously into a pheasant. The bird developed typical signs of botulism in 48 hours—dragging of the legs, paralysis, green diarrhea and hyperactivity of the eyes. Paralysis and other toxic signs continued for 3 days but the pheasant recovered.

Supernatant fluid of ground liver and spleen tissue was toxic to mice after heating to 56 C for 10 minutes but not after heating to 100 C for 30 minutes.

DISCUSSION

Botulism in pheasants has been reported by Dinter and Kull (1954) in Europe. In New York state about 8,000 pheasants died of botulism in 1957 (Cheatum, *et al.*[5]). Botulism has previously been suspected among penned pheasants in Wisconsin[1].

Type C botulism was first recorded in Wisconsin in 1944 when it was detected in meat scraps from a packing plant in the Green Bay area. Samples of the scraps used in mink food were submitted to the University of Wisconsin, Bacteriology Department (McCoy[7]). In 1957, Type C botulism toxin was isolated and identified from feed board scrapings at a mink ranch in Wisconsin. Various ingredients such as horse meat and fish were tested with inconclusive results[2].

Type C botulism has been known to infect wild ducks in Wisconsin[1]. In the summer of 1956, about 700 ducks died in the Green Bay area from a disease subsequently identified as Type C botulism by the Animal Disease Diagnostic Laboratory. Other migratory birds have been affected in the same area with varying degrees of severity.

The present study has shown that botulism was a cause of the pheasant mortality at the northeastern Wisconsin game farm. Pathological and toxicological observations support the diagnosis. Although spore-forming anaerobes were observed in thioglycolate broth inoculated with material from the in-

testinal tract and from the liver and spleen, the organisms were not studied.

Botulism can be produced in pheasants that consume carcasses of birds which have died of the disease. Dinter and Kull[6] and Bengston[4] have shown that maggots of blowflies *(Lucilia* and *Calliphora)* developing in spoiled flesh contain enough toxin to kill chickens and pheasants respectively. Masses of dead and decaying organic matter provide the culture medium in which the growth of the clostridia, producing botulism toxin, can occur. It appears that botulism outbreaks on pheasant raising establishments typically occur after a number of years of successful operation. During succeeding years, botulism occurs sporadically. This may be explained in one of two ways: (1) The microenvironment of the area involved is modified when the birds are introduced and held under relatively crowded conditions. Over a number of years, the accumulation of fecal material and spilled feed and its eventual decay may present conditions favorable for multiplication of the clostridial organisms already present in the soil. Its introduction into the carcass of a bird dead from other causes may then set the stage for an outbreak[3]; (2) another and probable explanation is that the botulism organism is carried to the farm by free-flying wild birds that come to feed with the pheasants. The outbreak then originates when a few birds that have ingested spores die from some other cause. The ingested organism proliferates after death, producing large quantities of toxin in the putrefying tissues that fatally intoxicate birds which peck at the carcass. If the carcasses are not removed promptly, the stage is set for the intoxication of increasing numbers of pheasants. The organism may spread to different pens through the dispersal of flies that breed in putrefying carcasses. Such carcasses were observed at the game farm by the authors. Pheasants probably ingested the toxin either by feeding upon the putrefying meat or fly larvae or adult flies. Since fatal intoxication depends upon dosage, removal of the exposed birds from the source by transferring them from the farm led to the recovery of many individuals.

ACKNOWLEDGMENT

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SUMMARY

The liver and spleen tissues from diseased pheasants were toxic to a healthy pheasant in which typical signs of botulism were induced.

Liver and spleen tissues from diseased pheasants produced a fatal toxemia of mice in 24 hours. The lethal toxin was specifically neutralized by *Cl. botulinum* Type C antitoxin. The toxin was inactivated by heating to 100 C for 30 minutes.

Bacterial and viral agents were not isolated from brain, liver, spleen and blood. Serological and pathological studies eliminated equine encephalomyelitis and Newcastle disease as etiological factors. Lead poisoning and DDT poisoning were eliminated on the basis of pathologic and epizootic grounds.

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5. Pennsylvania (Field Trial #4)	3.20	3.20	2.48	2.48
6. North Carolina	3.19	3.18	2.30	2.31
7. Mississippi	2.25	2.23	2.51	2.50
8. New York (9 weeks)	3.49	3.40	2.40	2.40
9. Rhode Island	2.49	2.34	2.34	2.40
10. Alabama (Test #2)	2.60	2.53	2.00	2.04
11. Connecticut	3.07	3.05	—	—
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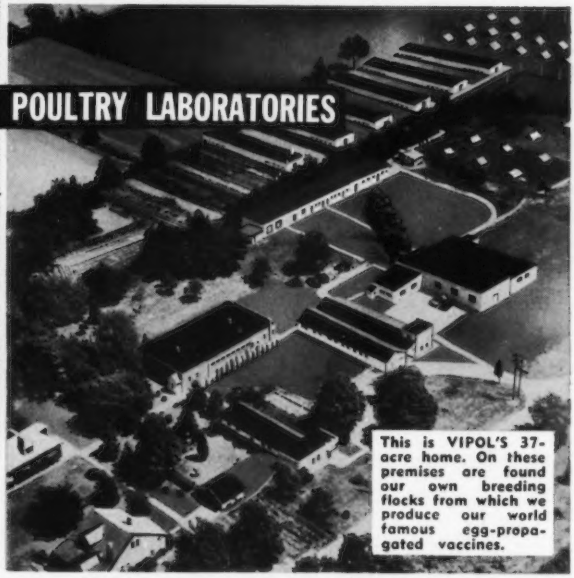

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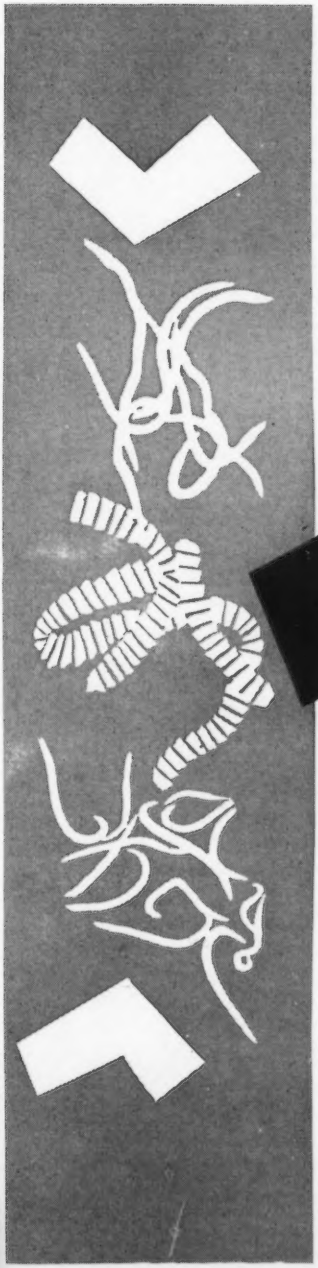

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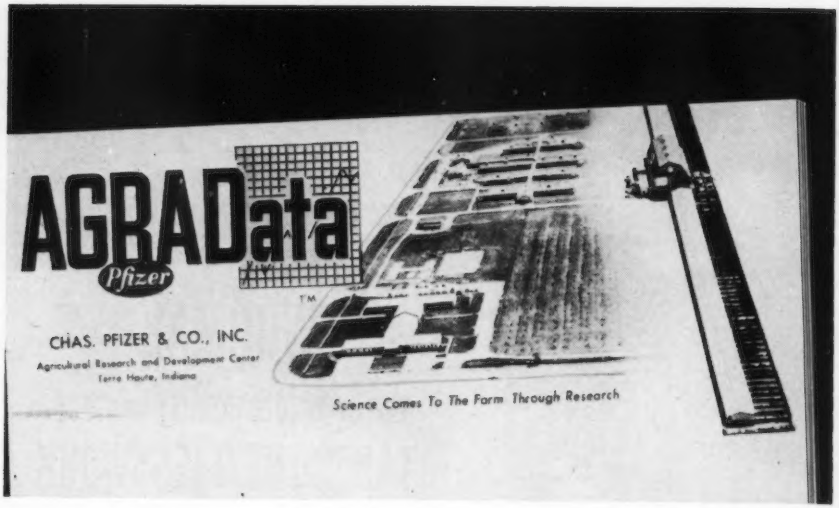

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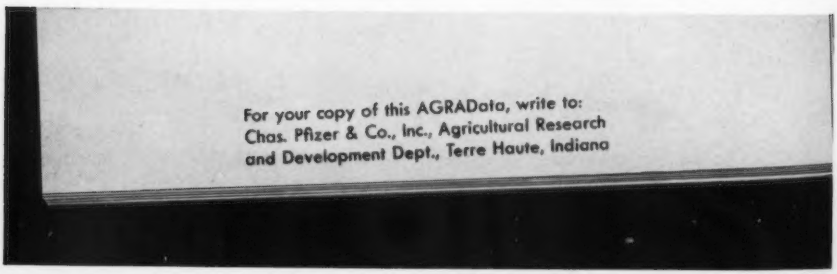
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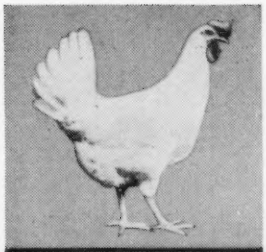

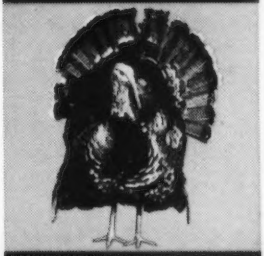

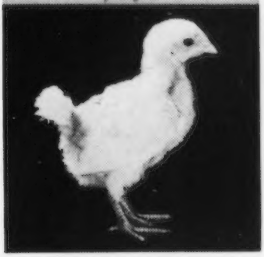

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DUOSTREP *is low in cost!* In the treatment of infectious sinusitis, *because of its specific action in this disease,* DUOSTREP is more economical to use than broad-spectrum antibiotics. For specific, effective, convenient treatment of infections sinusitis, use or recommend low-cost DUOSTREP Injectable Solution.

for treatment of infectious sinusitis

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